Regulation of ITGA6 and ITGB4 Integrin Genes by RUNX1 and Epigenetic Mechanisms

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BBiotech (Hons)

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Table of Contents

Declaration

Declarations of Originality

This thesis contains no material that has been accepted for a degree or diploma by the University of Tasmania or any other institution, except by way of background information and duly acknowledged in the thesis. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due acknowledgement is made in the text.

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Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

Use of human whole blood samples was approved by Human Research Ethics Committee (Tasmanian network), reference number: H8551.

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Contribution of Work

I assess my contribution to the work described in each chapter to be the following:

- Chapter 3, "Identifying Integrin Genes Regulated by RUNX1" 94%
    - ChIP-seq data analysis was undertaken by Kris Hardy at the University of Canberra.
    - pXP1-ITGB5 construct was cloned by a previous student, Paulynn Chin.
    - pXPG-ITGB4 was cloned by Jessica Phillips prior to this PhD.
- Chapter 4, "Regulation of *ITGB4* and *ITGA6* by RUNX1 in Myeloid Cells" 97%
    - Visualisation of ChIA-PET data was made possible by Kris Hardy at the University of Canberra.
    - pXPG-ITGB4 Del-2, pXPG-ITGB5 Del-1, pXPG-ITGB5 Del-2 and pXPG-ITGB5 Del-3 was cloned by Jessica Phillips prior to this PhD
- Chapter 5, "Epigenetic Regulation of *ITGB4* and *ITGA6*" 80%
    - Bisulphite sequencing of the ITGB4 promoter was conducted by Melissa Aubrey, University of Tasmania, as an undergraduate research project under the guidance of Jessica Phillips.
    - Bisulphite sequencing of the ITGB4 promoter in 5-aza-2'-deoxycytidine treated cells was conducted by Geoffrey Phillips under the guidance of Jessica Phillips.
    - Bisulphite sequencing of the ITGA6 promoter was conducted by Conor Cleary and Claudia Payne, University of Tasmania, as an undergraduate research project under the guidance of Geoffrey Phillips and Jessica Phillips.

This thesis is less than 100,000 words in length not including tables, figure legends and bibliographies.

Jessica Louise Phillips, BBiotech. Hons.

Statement of Co-Authorship

At the time of writing, the following publications have arisen from the data presented in this thesis:

Phillips JL, Taberlay PC, Woodworth AM, Hardy K, Brettingham-Moore KH, Dickinson JL and Holloway AF, Distinct mechanisms of regulation of the ITGA6 and ITGB4 genes by RUNX1 in myeloid cells. (Under review)

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List of Figures

List of Tables

Abbreviations

˚C	degrees Celsius
µg	microgram
µL	microliter
µM	micromolar
3'	three prime
5'	five prime
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
A	adenine
ABL	Abelson murine leukaemia
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
ANOVA	analysis of variance
APL	acute promyelocytic leukaemia
B-ALL	B-cell acute lymphoblastic leukaemia
BCL2	B-cell lymphoma 2
B-CLL	B-cell chronic lymphocytic leukaemia
BCR	breakpoint cluster region
bp	base pair
BSA	bovine albumin serum
C	cytosine
C/EBP	CCAAT/enhancer-binding protein
CBF	core binding factor
CBFB	core binding factor beta
CBP	CREB-binding protein
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
ChIA-PET	chromatin interaction analysis by paired-end tag sequencing
ChIP	chromatin immunoprecipitation
ChIP-seq	chromatin immunoprecipitation sequencing
CLL	chronic lymphocytic leukaemia
CML	chronic myeloid leukaemia
CMV	cytomegalovirus
CpG	5'-cytosine-phosphate-guanine-3'
DAC	5-aza-2’-deoxycytidine
DHS	deoxyribonuclease hypersensitive site
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide
DTT	dithiothreitol
ECM	extracellular matrix
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ENCODE	encyclopedia of DNA elements

ERG	ETS-related gene
ETO	eight twenty one
FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
FLI1	friend leukaemia integration 1
FLT3	fms-like tyrosine kinase 3
FOG1	friend of GATA 1
FPD	familial platelet disorder
FPD/AML	familial platelet disorder with a predisposition to AML
G	guanine
g	grams
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
G-CSF	granulocyte-colony stimulating factor
gDNA	genomic DNA
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
H3K27ac	histone H3 lysine 27 acetylation
H3K27me3	histone H3 lysine 27 trimethylation
H3K4me1	histone H3 lysine 4 monomethylation
H3K4me2	histone H3 lysine 4 dimethylation
H3K4me3	histone H3 lysine 4 trimethylation
H3K9me3	histone H3 lysine 9 trimethylation
HAT	histone acetyltransferase
HCl	hydrogen chloride
HDAC	histone deacetylase
HDM	histone demethylase
HF	high fidelity
HMT	histone methyltransferase
HRP	horseradish peroxidase
HSP	haematopoietic stem cell
IL	interleukin
ITGA	integrin alpha
ITGB	integrin beta
kb	kilobase
L	litre
LMO2	LIM domain only 2
log	logarithm
LYL1	lymphoblastic leukaemia-derived sequence 1
M	molar
M-CSF	macrophage colony-stimulating factor
$MgCl_2$	magnesium chloride
MK	megakaryocyte
mL	millilitre
MLL	mixed lineage leukaemia
mM	millimolar
MMC	macromolecular complex
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid

MYH11	myosin heavy chain 11
n	sample size/replicates
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NCBI	National Center for Biotechnology Information
N-CoR	nuclear receptor co-repressor
NEB	New England Biolabs
NF-κB	nuclear factor-κB
ng	nanogram
nm	nanometre
ns	not significant
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
PML	promyelocytic leukaemia
RARα	retinoic acid receptor alpha
RE	restriction enzyme
RGD	arginylglycylaspartic acid
RHD	runt homology domain
RhoD	rhodopsin
RNA	ribonucleic acid
RNA PolII	ribonucleic acid polymerase 2
rpm	revolution per minute
RPMI	Roswell Park Memorial Institute
RT-qPCR	real-time reverse transcription quantitative polymerase chain reaction
RUNX	Runt-related transcription factor
SAP30BP	SAP30 binding protein
SCL	stem cell leukaemia
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of mean
siRNA	small interfering ribonucleic acid
STAT	signal transducer and activator of transcription
SV40	Simian vacuolating virus 40
T	thymine
TCR	T-cell receptor
TE	tris-ethylenediaminetetraacetic acid
TET	ten-eleven-translocation
TI	total input
Tris	Tris(hydroxymethyl)aminomethane
TSA	Trichostatin A
UCSC	University of California Santa Cruz
UTR	untranslated region
V	volts
VEGF	vascular endothelial growth factor
VLA	very late activation antigen
x *g*	gravitational acceleration
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Abstract

Disruption to regulatory mechanisms controlling gene expression is a hallmark of leukaemia, with disruption to transcription factors being one of the most prevalent. By identifying the gene expression profile under the control of these transcription factors, and understanding how the target genes are regulated, critical insight can be gained into the role of these transcription factors in haematopoiesis, as well as their role in leukaemia development. Evidence presented here suggests that the RUNX1 transcription factor regulates the expression of the α6β4 integrin receptor in haematopoietic cells by controlling the integrin genes ITGA6 and ITGB4. Engagement of integrin receptors with extracellular matrix components of the bone marrow and haematopoietic tissues plays an essential role in haematopoiesis. Integrin expression is also altered in many leukaemias, however the regulation of integrin gene expression both in normal and disease states has remained largely unexplored.

Data presented here identified *ITGA6* and *ITGB4* as novel target genes of the RUNX1 transcription factor in myeloid cells. RUNX1 was found to bind to the promoter regions of *ITGA6* and *ITGB4* in myeloid cells in ChIP assays. Furthermore, RUNX1 had a functional effect on both of the promoters in reporter assays. RUNX1 increased the activity of both promoters, while RUNX1-ETO, which is produced by a common chromosomal translocation in leukaemic cells, repressed promoter activity, consistent with its well-characterised role as a transcriptional repressor.

While RUNX1 is commonly described as a sequence-specific DNA binding protein that binds to the consensus motif TGT/cGGT, it is becoming evident that the regulation of genes by RUNX1 is more complex than this and RUNX1 can regulate its target genes through a variety of mechanisms. Evidence presented here suggests that RUNX1 regulates the ITGA6 and ITGB4 integrin genes via two distinct mechanisms. RUNX1 was found to regulate the ITGA6 promoter through a consensus RUNX1 binding motif and RUNX1 activation of the promoter was dependent on this motif, in keeping with the traditional model of RUNX1 function described in the literature. In contrast, RUNX1 does not target the ITGB4 promoter through a consensus sequence motif and may be recruited indirectly to the promoter by other haematopoietic transcription factors.

Furthermore, the data presented here suggest that efficient regulation of the ITGB4 gene may require interactions between the promoter and an upstream enhancer. RUNX1 was also found to interact with the ITGB4 enhancer, and similarly to the promoter, these interactions do not require a RUNX1 consensus binding motif and may involve recruitment by other transcription factors. Recent evidence suggests that the traditional model of RUNX1 function through a consensus binding motif may represent only a small proportion of RUNX1 target genes. Genome-wide analysis suggests that a significant proportion of RUNX1 recruitment to DNA occurs in the absence of consensus binding motifs, as shown here for *ITGB4*.

To regulate gene expression, transcription factors must operate in the context of the nuclear chromatin environment. RUNX1 influence on gene expression is therefore also dependent on the chromatin environment at its target genes and its interactions with this environment. In the present study, epigenetic mechanisms were also found to contribute to the regulation of ITGA6 and ITGB4 gene expression in myeloid cell lines. *ITGB4* expression was inversely correlated with DNA methylation of a large CpG island located at the promoter in KG-1a and Kasumi-1 myeloid cells. Furthermore, low levels of histone H3 and high levels of histone H3 acetylation at both ITGA6 and ITGB4 promoter regions was associated with higher expression of the genes in these cells. Expression of the ITGA6 and ITGB4 genes is likely to be a result of the interplay between transcription and epigenetic factors and in support of this, data presented here show that despite the presence of RUNX1 in KG-1a cells, *ITGB4* is expressed at very low levels in these cells possibly due to high levels of DNA methylation at the promoter.

This study has advanced our understanding of the mechanisms by which RUNX1 regulates its target genes and has identified distinct molecular mechanisms by which it operates. These findings may also be relevant to the mechanisms by which other transcription factors operate. Additionally, these findings suggest that RUNX1 disruption in leukaemia may have different effects on its target genes depending on how they are regulated normally by RUNX1. Additional studies are therefore required to further dissect the mechanisms by which RUNX1 regulates its target genes, and to further elucidate the repertoire of RUNX1 controlled genes. In addition, this study has provided insight into the regulation of integrin genes in myeloid cells, which is likely to have relevance to the regulation of these genes in other cell types and disease states.

Chapter 1

Introduction

1.1 Haematopoiesis and Leukaemia

1.1.1 Haematopoiesis

Haematopoiesis, in an adult, is a process which occurs primarily in the bone marrow and results in the development of immature haematopoietic progenitor cells into mature differentiated haematopoietic cells (Speck and Gilliland 2002). Since mature haematopoietic cells have a relatively short life-span, haematopoietic stem cells in the bone marrow are responsible for replenishing these cells (Orkin and Zon 2008, Speck and Gilliland 2002). Haematopoietic stem cells have the ability to regenerate, using a process called self-renewal, and have the potential to differentiate into haematopoietic progenitors which later differentiate into the mature cells of the haematopoietic system (Orkin 1995, Orkin 2000). As the haematopoietic progenitors differentiate, the cells become committed to maturation along specific lineage pathways (Orkin 2000, Orkin and Zon 2008). Haematopoiesis is therefore important for maintaining the optimum number of mature functional haematopoietic cells for homeostasis, efficient delivery of oxygen and protection against infection (Speck and Gilliland 2002). Haematopoiesis is tightly controlled through the complex interplay between extrinsic growth factors and cytokines that act on the cells, modulating the intrinsic signal transduction pathways and cell specific gene expression programs (Broxmeyer 2001, Cantor and Orkin 2002, Georgopoulos 2002, Kaushansky 2006, Orkin and Zon 2008).

1.1.2 Leukaemia

Leukaemias arise as a result of abnormal and disordered haematopoiesis due to altered regulation of the mechanisms controlling haematopoietic cell development (Speck and Gilliland 2002). Leukaemia is classified into groups based on the clinical course: acute or chronic, and the lineage commitment: lymphoid or myeloid (Burmeister and Thiel 2001). Leukaemias can also be sub-classified based on other characteristics such as morphology, cytochemistry, immunophenotype and genetic information (Vardiman *et al.*

2009, Walter *et al.* 2013). The treatment of individuals with leukaemia varies depending on the classification of the leukaemia and other factors such as low or high-risk state of disease, the health of the affected individual and the age of the individual (Burnett *et al.* 2011, Cripe and Hinton 2000, Wei *et al.* 2010). The development of leukaemia occurs when the haematopoietic progenitor cells acquire mutations which provide the cells with a survival and/or proliferative advantage, and impair differentiation and apoptosis (Rubnitz *et al.* 2010, Speck and Gilliland 2002).

In the early 2000's the two-hit model hypothesis for the development of leukaemia was proposed (Gilliland and Griffin 2002). It was suggested that in order for leukaemia to develop, a haematopoietic stem cell or progenitor cell accumulates genomic alterations which affect genes involved in regulating cell proliferation, cell death and cell differentiation (Gilliland and Griffin 2002, Renneville *et al.* 2008). These genetic alterations transform the haematopoietic cell into what is now recognised as a leukaemic stem cell which retains the ability of self-renewal (Gilliland and Griffin 2002, Renneville *et al.* 2008). According to the two-hit model, these mutations to the haematopoietic stem cell are divided into two classes: class I mutations activate signal transduction pathways which provide the cell with a proliferation or survival advantage and class II mutations affect transcription factors or factors of the transcriptional complex, which block myeloid differentiation and provide self-renewability (Döhner and Döhner 2008, Gilliland and Griffin 2002, Renneville *et al.* 2008). In support of the two-hit hypothesis, studies have shown the co-occurrence of these two classes of mutations in human leukaemias. For example, individuals with acute myeloid leukaemia (AML) with a class II mutation, such as to the RUNX1 gene, have a higher frequency of mutations in class I genes encoding signal transduction molecules, such as *FLT3*, *N-RAS*, *PTPN11* and *NF1*, resulting in hyperactivation of receptor tyrosine kinase-RAS signalling pathways (Niimi *et al.* 2006, Tang *et al.* 2009).

However, recent research suggests that the development of leukaemia is more complex than this and that additional classes of mutations are required which affect epigenetic modification, cell adhesion and DNA repair. A recent study found that around 50% of individuals with AML had at least two mutations in genes commonly disrupted in leukaemia (Rocquain *et al.* 2010). These genes were separated into four different classes: class I (transcription factors), which include *RUNX1* and *TET2* mutations, and cause clonal dominance of haematopoietic stem cells; class II (epigenetic), which include

ASXL1 and *NPM1* mutations, and promote either primary or secondary AML; class III (signal transduction), which include *CBL*, *FLT3*, *JAK2* and *RAS* mutations, and affect signalling pathways and proliferation, and class IV, which include *IDH1*, *IDH2* and *WT1*, and are exclusive mutations but are found to co-occur with mutations of genes in other classes and are associated with the acute phase of leukaemia (Rocquain *et al.* 2010). It was suggested that at least one mutation in each class was required for the development of AML (Rocquain *et al.* 2010).

Along similar lines, Thiede (2012) proposed that as well as the class I and class II mutations from the two-hit model, which promote cell proliferation and survival, and block differentiation, there are another three classes of mutations that are required for the development of leukaemia: class III mutations which affect epigenetic modifications; class IV mutations which affect cell adhesion and cell-cell interaction; and class V which affect DNA repair/RNA splicing (Thiede 2012). In contrast, Kitamura *et al.* (2015) recently proposed that there are four classes of cellular phenotypes caused by mutations which lead to the development of leukaemia: induction of proliferation (class I), survival or block of differentiation (class II), block of differentiation (class III) and immortalisation (class IV) (Kitamura *et al.* 2015). It was proposed that a combination of mutation-induced cellular phenotypes would induce acute leukaemia and can determine the disease phenotypes of leukaemia (Kitamura *et al.* 2015). This revised model focuses on the cellular phenotypes induced by gene mutations rather than the categories of the genes that are mutated, and it is suggested that this is a better representation of the molecular basis of leukaemogenesis (Kitamura *et al.* 2015).

While the details of these proposed models differ, the overarching principle is that the development of leukaemia is complex and multiple genetic disruptions are required, which increase proliferation, block apoptosis, block differentiation and immortalise the haematopoietic cells. Further, a recurrent theme is that mutations to genes encoding transcriptional regulators are a common underlying cause of the disruption to haematopoiesis that occurs in leukaemia.

1.1.3 Transcriptional Regulation of Haematopoiesis

Conditional knock-out/expression studies conducted in mice have identified many of the transcription factors required for haematopoiesis, and not surprisingly, many of these are

also commonly disrupted in leukaemia (Figure 1.1). Through these studies, transcription factors such as PU.1, GATA1 and C/EBPα have been demonstrated to be required for specific cell lineages (Pevny *et al.* 1991, Scott *et al.* 1994, Zhang *et al.* 1997). *Gata1* is required for normal differentiation of erythroid cells, as mice deficient in *Gata1* lack mature red blood cells (Pevny *et al.* 1991). Furthermore, mice deficient in *Pu.1* also showed disrupted erythroid cell differentiation, but additionally displayed defects in the generation of progenitors for B and T lymphocytes, monocytes and granulocytes (Scott *et al.* 1994). In addition, mice deficient in *C/ebpα* lack mature neutrophils and eosinophils in the blood and foetal liver, but other haematopoietic lineages were not affected (Zhang *et al.* 1997).

Transcription factors such as SCL, GATA2 and LMO2 have been identified as essential for the emergence of haematopoietic stem cells. A study by Tsai *et al* (1994) determined that *Gata2* is required for the normal development of mice as *Gata-2* knock-out mice were found to die by embryonic days 10-11. On further analysis, it was determined that *Gata-2* was important for the emergence of haematopoietic stem and progenitor cells as *Gata2* chimeric mice embryos were deficient in these cells (Tsai *et al.* 1994). Similar to *Gata-2*, *Lmo2* knock-out mice died at embryonic day 10.5 and also lacked yolk sac erythropoiesis (Warren *et al.* 1994). Additionally, absence of the transcription factor Scl in mice displayed a similar phenotype to *Lmo2* knock-out mice with embryonic lethality at days 8.5-10.5 and a lack of nucleated red blood cells (Shivdasani *et al.* 1995).

Furthermore, MLL, RUNX1 and TEL/ETV6 genes are also important for the emergence of haematopoietic stem cells. *Mll*, *Runx1* and *Etv6* knock-outs were found to be embryonic lethal (Hess *et al.* 1997, Okuda *et al.* 1996, Wang *et al.* 1998). *Mll* knock-out mice had reduced yolk sac haematopoiesis and *Mll* was found to be required for maintaining the correct number of haematopoietic progenitors as well as the appropriate differentiation of these cells (Hess *et al.* 1997). Additionally, the *Etv6* transcription factor was found to be essential for the establishment of haematopoiesis of all lineages in the bone marrow (Wang *et al.* 1998). *Runx1* knock-out mice, which showed a more severe phenotype, lacked foetal liver haematopoiesis and myeloid/erythroid progenitors were absent, suggesting that *Runx1* is required for definitive haematopoiesis of all lineages (Okuda *et al.* 1996).

Not surprisingly, the majority of these transcription factors identified to be essential for haematopoiesis are targets of somatic mutations and/or chromosomal translocations in leukaemia; suggesting disruption to these transcription factor genes predisposes an individual to leukaemia (Orkin and Zon 2008).

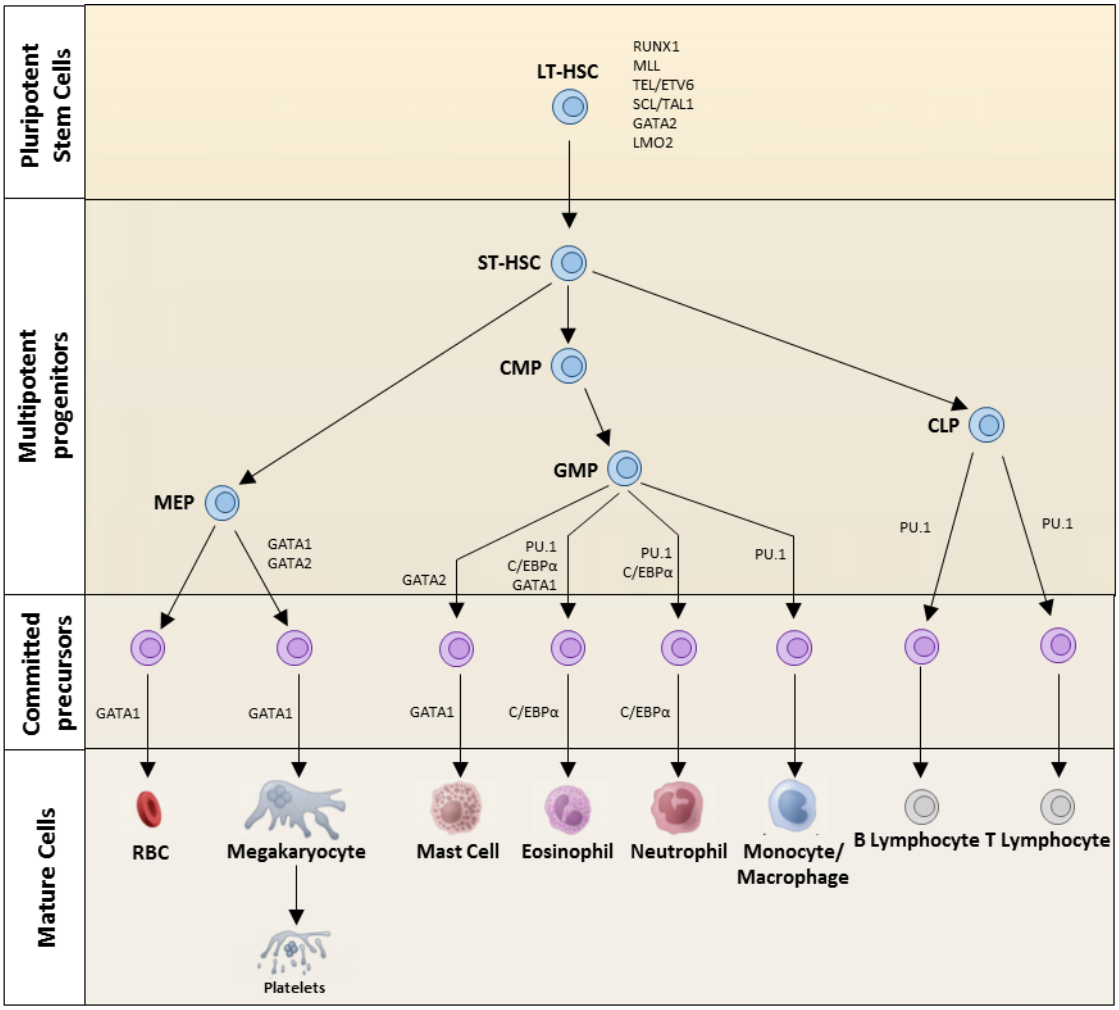


Figure 1.1 – Role of transcription factors in haematopoiesis. The stages at which transcription factors are important for haematopoietic development are shown. Absence of these transcription factors have been shown to block haematopoietic development in knock-out mice studies. Adapted from Orkin *et al*. (2008).
Abbreviations: LT-HSC = long-term haematopoietic stem cell; ST-HSC = short-term haematopoietic stem cell; CMP = common myeloid progenitor; CLP = common lymphoid progenitor; MEP = megakaryocyte/erythroid progenitor; GMP = granulocyte/macrophage progenitor; RBCs = red blood cells.

1.2 RUNX1

1.2.1 Runt-Related Transcription Factors

The Runt-related transcription factors (RUNX), which belong to a small group of heterodimeric transcription factors known as the core-binding factors (CBFs), play important roles in cellular development and differentiation (Goyama and Mulloy 2011, Ito 2004, Speck and Gilliland 2002, Wang *et al.* 2010). The RUNX family comprises three evolutionary conserved genes, RUNX1, RUNX2 and RUNX3, which encode an alpha DNA binding subunit that forms part of a CBF complex (Blyth *et al.* 2005, De Braekeleer *et al.* 2011, Goyama and Mulloy 2011, Speck and Gilliland 2002, Wang *et al.* 2010). A single gene, CBFB, encodes the non-DNA binding beta subunit, CBFβ, which is important for the stability of the CBF complex, high affinity DNA binding of the RUNX subunits and protection of the RUNX subunits, particularly RUNX1, against degradation (Blyth *et al.* 2009, Goyama and Mulloy 2011, Huang *et al.* 2001, Kagoshima *et al.* 1996). The RUNX transcription factors all share a region of high sequence homology at the N-terminus, called the Runt domain or Runt homology domain (RHD), which is responsible for heterodimerisation with CBFβ and DNA binding (Blyth *et al.* 2005, Goyama and Mulloy 2011, Kagoshima *et al.* 1996). This 128 amino acid Runt domain was first identified in the *Drosophila* Runt gene, which was found to be important for early embryonic segmentation (Blyth *et al.* 2005, Gergen and Butler 1988). The C-terminus of the RUNX transcription factors is less conserved than the Runt domain and contains inhibitory and activation domains that interact with transcriptional co-repressors and co-activators (Blyth *et al.* 2005). The RUNX transcription factors therefore can have a positive or negative influence on the transcription of their target genes.

The RUNX1 gene, which is located on chromosome 21, is required for definitive haematopoiesis and is a common target of chromosomal and genetic alterations in leukaemia (Blyth *et al.* 2005, Goyama and Mulloy 2011, Ito 2004, Okuda *et al.* 1996, Speck and Gilliland 2002, Wang *et al.* 1996, Yamagata *et al.* 2005). *RUNX2*, which is located on chromosome 6, is involved in osteogenesis and has been found to play a role in bone metastasis in breast and prostate cancer (Akech *et al.* 2009, Barnes *et al.* 2003, Barnes *et al.* 2004, Komori *et al.* 1997, Otto *et al.* 1997). *RUNX3*, which is located on chromosome 1, is important for neurogenesis, thymopoiesis and maintenance of gut epithelium, and has been associated with the development of gliomas and gastric cancer

(Levanon *et al.* 2001, Levanon *et al.* 2002, Li *et al.* 2002, Mei *et al.* 2011, Mueller *et al.* 2007, Woolf *et al.* 2003).

1.2.2 The RUNX1 Transcription Factor

The RUNX1 gene, also known as *AML1*, *CBFA2* and *PEBP2aB*, was first identified from the frequently occurring chromosomal translocation t(8;21) in AML (Miyoshi *et al.* 1991). Further studies involving knock-out mice determined that RUNX1 was important for haematopoiesis. These studies found mice homozygous for mutant *Runx1* died between embryonic days 11.5-12.5 due to haemorrhaging in the central nervous system (Okuda *et al.* 1996, Wang *et al.* 1996). The homozygous mice also lacked foetal liver haematopoiesis and were deficient in myeloid/erythroid progenitors, suggesting that *RUNX1* is required for definitive haematopoiesis (Okuda *et al.* 1996, Wang *et al.* 1996). Further studies discovered that CBFβ is required for RUNX1 function and therefore, is also important for definitive haematopoiesis. In these studies, knock-out *Cbfb* mice were found to exhibit the same phenotype as the *Runx1* knock-out mice (Sasaki *et al.* 1996, Wang *et al.* 1996). These mice also died at embryonic days 11.5-14.5 due to haemorrhaging in the central nervous system and the mice also lacked definitive haematopoiesis in the foetal liver (Sasaki *et al.* 1996, Wang *et al.* 1996)

The expression of *RUNX1* is controlled by two promoters, distal (P1) and proximal (P2), which direct transcription of RUNX1 isoforms (Blyth *et al.* 2005, De Braekeleer *et al.* 2011, Ghozi *et al.* 1996, Lam and Zhang 2012, Miyoshi *et al.* 1995). Three RUNX1 isoforms have been described: RUNX1a, RUNX1b and RUNX1c, which all possess the Runt domain in the N-terminal region (De Braekeleer *et al.* 2011, Lam and Zhang 2012, Miyoshi *et al.* 1995). The RUNX1a isoform, consisting of 250 amino acids, lacks the transcriptional regulatory domains located in the C-terminal region and therefore only possesses the DNA-binding N-terminal region of RUNX1 (De Braekeleer *et al.* 2011, Lam and Zhang 2012, Miyoshi *et al.* 1995). The RUNX1b isoform, consisting of 453 amino acids, contains both the N-terminal region and C-terminal transcriptional regulatory domains (De Braekeleer *et al.* 2011, Lam and Zhang 2012, Miyoshi *et al.* 1995). The RUNX1c isoform, consisting of 480 amino acids, is the longest RUNX1 isoform and only differs from RUNX1b by 32 amino acids in the N-terminal region (De Braekeleer *et al.* 2011, Lam and Zhang 2012, Miyoshi *et al.* 1995). RUNX1c and RUNX1b both possess identical C-terminal regions and RUNX1a and RUNX1b both

share the same N-terminal region (De Braekeleer *et al.* 2011, Lam and Zhang 2012, Miyoshi *et al.* 1995). The expression of the RUNX1 isoforms has been shown to play an important and non-redundant role in haematopoiesis (Bee *et al.* 2010, Challen and Goodell 2010, Fujita *et al.* 2001, Li *et al.* 2002, Pozner *et al.* 2007, Ran *et al.* 2013, Sroczynska *et al.* 2009).

As well as being transcriptionally regulated, RUNX1 is regulated by post-translational changes such as phosphorylation and methylation (Lam and Zhang 2012, Wang *et al.* 2009). Phosphorylation at 2-3 serine residues of RUNX1 is generally associated with RUNX1 activation of transcription (Guo and Friedman 2011, Imai *et al.* 2004, Lam and Zhang 2012). Phosphorylation of RUNX1 increases the ability of RUNX1 to activate the transcription of target genes by decreasing RUNX1 interactions with transcriptional co-repressors (Guo and Friedman 2011, Imai *et al.* 2004, Lam and Zhang 2012). However, phosphorylation of RUNX1 also results in the time-dependent degradation of RUNX1, therefore increased activation of RUNX1 by phosphorylation is transient (Biggs *et al.* 2006, Imai *et al.* 2004). Methylation at two arginine residues of RUNX1 has also been found to contribute to RUNX1 activation of transcription by causing dissociation from transcriptional co-repressors (Zhao *et al.* 2008).

1.2.3 RUNX1 in Leukaemia

RUNX1 is a frequent target of genetic alteration in leukaemia (Blyth *et al.* 2005, Michaud *et al.* 2008, Speck and Gilliland 2002), with chromosomal translocations, mutations and gene amplification of *RUNX1* all observed in individuals with the disease (Dal Cin *et al.* 2001, Harewood *et al.* 2003, Miyoshi *et al.* 1991, Niini *et al.* 2000, Osato *et al.* 1999, Preudhomme *et al.* 2009, Song *et al.* 1999, Streubel *et al.* 2001). It is proposed that these alterations to *RUNX1* predispose individuals to leukaemia.

1.2.3.1 RUNX1 Chromosomal Translocations

Chromosomal translocations involving the RUNX1 gene were the first alterations to *RUNX1* detected in individuals with leukaemia. *RUNX1* is implicated in approximately 55 different translocations, but only 21 have been completely characterised (De Braekeleer *et al.* 2011). Of these, the most common are t(8;21)(q22;q22) which is observed in approximately 12% of AML and 40% of the M2 subtype of AML (Peterson

and Zhang 2004), and t(12;21)(p13;q22) which is observed in 20-25% of paediatric acute lymphoblastic leukaemia (ALL) (Liang *et al.* 1996, Romana *et al.* 1995).

The t(8;21) chromosomal translocation was the first translocation involving the RUNX1 gene to be discovered (Blyth *et al.* 2005). This translocation was first identified in an individual with acute leukaemia in 1973 (Rowley 1973), and led to the discovery of *RUNX1* through the cloning of the gene on chromosome 21 involved in the t(8;21) chromosomal translocation (Miyoshi *et al.* 1991). In this study, the RUNX1 gene was found to be rearranged in individuals with AML with breakpoints of the gene in 16 out of 21 individuals clustered within the same region of *RUNX1* (Miyoshi *et al.* 1991). Interestingly, detailed analysis of 3 of those individuals found the breakpoints to occur in the same intron of the RUNX1 gene, suggesting that all t(8;21) breakpoints most likely occur in that intron (Miyoshi *et al.* 1991).

The other gene involved in the t(8;21) chromosomal translocation on chromosome 8 was subsequently discovered using a similar cloning method (Miyoshi *et al.* 1993). Miyoshi *et al.* (1993) discovered the novel MTG8 gene, also known as Eight Twenty One (ETO). Further analysis determined the chromosomal translocation encodes a RUNX1-ETO fusion transcript, which produces a RUNX1-ETO fusion protein. The fusion protein was found to contain the last 575 amino acids of the 604 amino acid ETO protein and the first 177 amino acids of the RUNX1 protein containing the DNA binding Runt domain (Figure 1.2A) (Miyoshi *et al.* 1993).

Further studies indicate that the RUNX1-ETO protein mainly acts as a dominant negative inhibitor of RUNX1 function. This was shown in knock-in mouse studies in which heterozygous mice died as embryos due to haemorrhaging in the central nervous system and foetal liver haematopoiesis was absent, although these mice possessed a functional copy of one Runx1 gene (Yergeau *et al.* 1997). RUNX1-ETO has also been shown to bind to RUNX1 target genes (Frank *et al.* 1995, Westendorf *et al.* 1998) since the DNA binding domain of RUNX1 is retained in the fusion protein. RUNX1 normally interacts with transcriptional co-activators to activate gene expression (Kitabayashi *et al.* 1998), however, due to the replacement of the transactivation domain of RUNX1 with ETO, expression of target genes is generally repressed due to interactions with repressive co-factors (Lutterbach *et al.* 1998, Wang *et al.* 1998). RUNX1-ETO therefore causes genome-wide changes in gene expression (Ptasinska *et al.* 2012), disrupting expression

of RUNX1 target genes and leading to inhibition of differentiation (Westendorf *et al.* 1998), inhibition of apoptosis (Klampfer *et al.* 1996, Matsushita *et al.* 1999) and increased haematopoietic progenitor/stem cell proliferation (Mulloy *et al.* 2002, Okuda *et al.* 1998).

Another common chromosomal translocation observed in leukaemia is t(12;21), which results in an ETV6-RUNX1 fusion protein. Involvement of the RUNX1 and ETV6 genes in this translocation was initially identified in two cases of childhood pre-B cell ALL (Golub *et al.* 1995). However, a subsequent study determined that there is a high frequency of this chromosomal translocation in childhood B-lineage ALL and the RUNX1-ETV6 fusion protein was detected in at least 16% of 121 individuals examined in one particular study (Romana *et al.* 1995).

The t(12;21) translocation results in the fusion of most of RUNX1, including the DNA binding domain and transactivation domain, to the 5' end of the ETV6 transcription factor, including the helix-loop-helix domain but not the DNA binding domain of the protein (Figure 1.2A) (Golub *et al.* 1995). Similar to the RUNX1-ETO fusion protein, ETV6-RUNX1 has been described as a transcriptional repressor (Chakrabarti and Nucifora 1999, Fenrick *et al.* 1999, Guidez *et al.* 2000). Since the RUNX1 DNA binding domain is retained in this fusion protein, ETV6-RUNX1 generally represses expression of RUNX1 target genes (Fears *et al.* 1997, Hiebert *et al.* 1996).

Additionally to RUNX1 chromosomal translocations, the CBFβ subunit, which heterodimerises with RUNX1, is also involved in a common chromosomal alteration observed in adult AML and paediatric ALL, known as inv(16), which produces a CBFβ-MYH11 fusion protein (Blyth *et al.* 2005, Lutterbach *et al.* 1999, Shurtleff *et al.* 1995). The CBFβ-MYH11 fusion protein blocks haematopoiesis and is thought to do so by disrupting normal RUNX1 activity (Castilla *et al.* 1996, Kanno *et al.* 1998, Kundu and Liu 2001).

1.2.3.2 Mutations in RUNX1

Since the discovery of RUNX1 chromosomal translocations and the importance of RUNX1 in haematopoiesis, mutations to the RUNX1 gene were subsequently identified as a common occurrence in leukaemia. A study by Osato *et al.* (1999) analysed 160 individuals with leukaemia for mutations in the RUNX1 gene. In six of these individuals,

silent, heterozygous missense, biallelic nonsense or frameshift mutations were identified. The mutations were found to be clustered within a region of the gene encoding the DNA binding Runt domain of RUNX1. The missense and biallelic mutations were associated with RUNX1 loss-of-function, suggesting that the mutations to *RUNX1* could play a role in the development of leukaemia (Osato *et al.* 1999). Similarly, a study by Tang *et al.* (2009) discovered 63 distinct *RUNX1* mutations in 62 of 470 adult patients with de novo AML. Of the 63 mutations, 7 were nonsense mutations, 24 were missense mutations, 28 were frame-shift mutations and 4 were in-frame mutations (Figure 1.2) (Tang *et al.* 2009). Interestingly, most of the mutations occurred in either the DNA binding Runt domain or the transactivation domain of RUNX1 (Tang *et al.* 2009).

In support of these studies, individuals with familial platelet disorder with a predisposition to AML (FPD/AML) are predisposed to AML due to haploinsufficiency of *RUNX1* as a result of genetic mutations (Preudhomme *et al.* 2009, Song *et al.* 1999). Studies have shown that individuals with FPD/AML possess a mutation to one allele of the RUNX1 gene which causes the platelet defect and additional mutations to *RUNX1* can then contribute to the development of leukaemia (Preudhomme *et al.* 2009, Song *et al.* 1999).

In more recent studies, it has been found that *RUNX1* mutations are associated with lower complete remission rates, shorter overall survival and resistance to therapy in AML (Gaidzik *et al.* 2011, Mendler *et al.* 2012, Schnittger *et al.* 2011, Tang *et al.* 2009). A study has found that individuals with *RUNX1* mutations have a significantly lower complete remission rate, shorter disease-free survival and overall survival than those individuals without *RUNX1* mutations (Tang *et al.* 2009). These findings have also been supported by other studies, which also found a correlation between *RUNX1* mutations and lower remission rates, disease-free survival and overall survival in AML (Gaidzik *et al.* 2011, Mendler *et al.* 2012, Schnittger *et al.* 2011). In addition, *RUNX1* mutations have also been associated with resistance to therapy. A study has shown that *RUNX1* mutations in individuals with AML is associated with resistance to chemotherapy (Gaidzik *et al.* 2011). *RUNX1* mutations were identified in 53 out of 945 individuals with AML and those patients were shown to have lower complete remission rates, higher refractory disease rates and higher early/hypoplastic death rates in response to chemotherapy compared to individuals with no *RUNX1* mutations (Gaidzik *et al.* 2011).

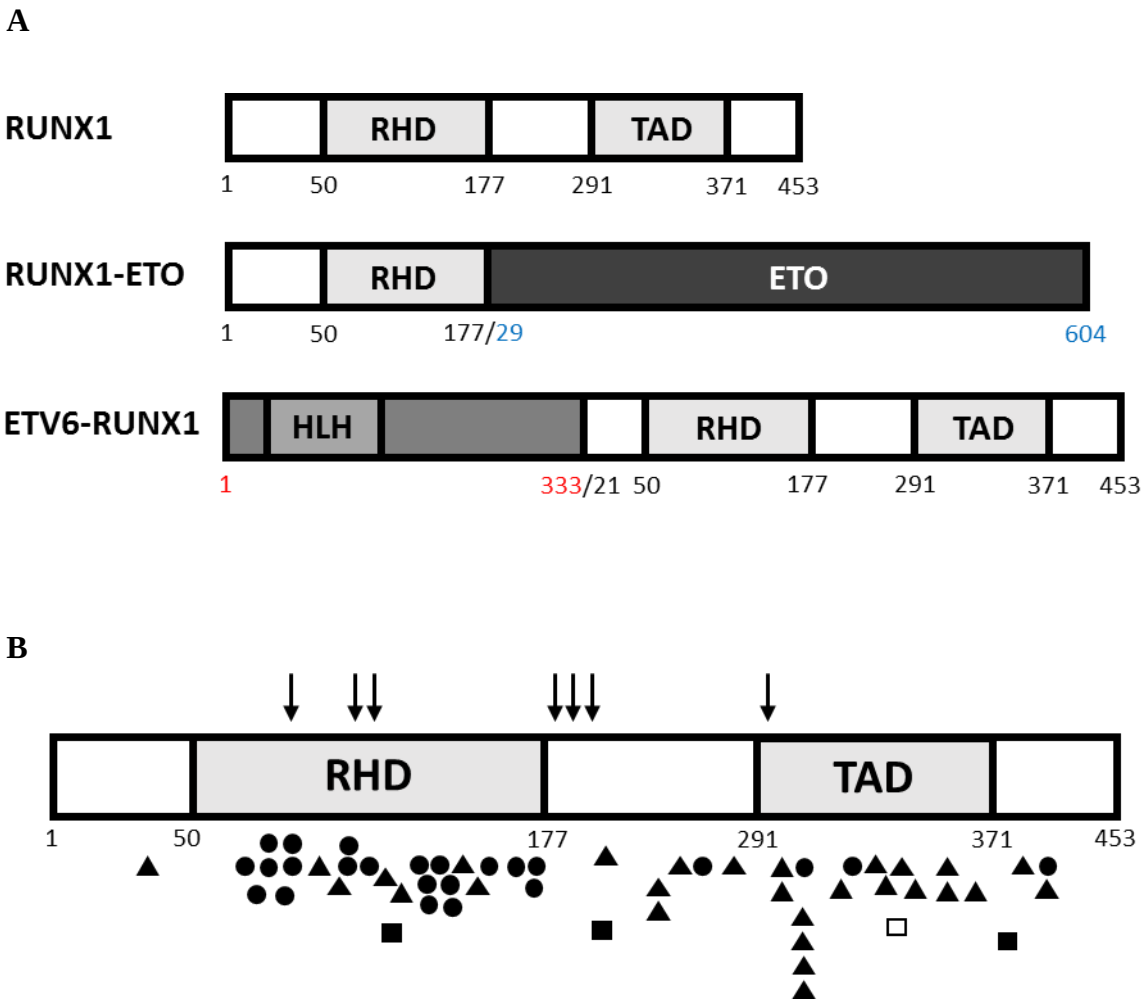


Figure 1.2 – Common chromosomal translocations and mutations in leukaemia. A) Schematic representation of RUNX1 protein, containing the Runt homology domain (RHD) and transactivation domain (TAD), RUNX1-ETO fusion protein produced from the t(8;21) chromosomal translocation and ETV6-RUNX1 fusion protein produced from the t(12;21) chromosomal translocation, containing the helix-loop-helix (HLH) domain of ETV6. Numbering of amino acids for RUNX1 (black), ETO (blue) and ETV6 (red) proteins are shown. **B)** Schematic of the RUNX1 protein showing the location of 63 distinct mutations identified in 62 individuals with de novo AML (Tang *et al.* 2009). Missense mutations (circles), frameshift mutations (triangles) and in-frame mutations (squares), including insertions (black squares) and a deletion (white square), are shown. The point at which the stop codon for RUNX1 is shifted due to nonsense mutations are shown by the arrows. The numbering of the amino acids relative to the Runt homology domain (RHD) and transactivation domain (TAD) of the RUNX1 protein is shown. Adapted from Tang *et al* (2009).

1.2.3.3 RUNX1 Gene Amplification

As well as decreased activity of RUNX1 caused by mutations or chromosomal translocations to the RUNX1 gene, increased expression of *RUNX1* caused by polysomy of chromosome 21, increased copies of the RUNX1 gene or deregulation of the RUNX1 promoter, has also been suggested as a contributing factor to the development of leukaemia (Mikhail *et al.* 2002, Mikhail *et al.* 2006). Amplification of *RUNX1* has been observed in individuals with ALL, particularly childhood ALL (Dal Cin *et al.* 2001, Harewood *et al.* 2003, Mikhail *et al.* 2002, Niini *et al.* 2000), and it has been suggested that the increased dosage of *RUNX1* in individuals with Down Syndrome, due to trisomy 21, could contribute to the development of leukaemia in those individuals (Blyth *et al.* 2005, Ito 2004, Yanagida *et al.* 2005).

The leukaemogenesis of *RUNX1* amplification has been supported by *in vivo* studies using mice predisposed to developing lymphoma or leukaemia (Wotton *et al.* 2002, Yanagida *et al.* 2005). In a study by Wotten *et al.* (2002), CD2-MYC mice, which are mice that develop lymphomas due to proviral insertional mutagenesis of the *Myc* gene (Stewart *et al.* 1993), were engineered to overexpress the Runx1 gene. *Runx1* was found to act as a dominant oncogene with *Myc* to cause the development of T-cell lymphoma in the transgenic mice (Wotton *et al.* 2002). In another study, BXH2 mice, which are mice that develop myeloid leukaemia over time, were also engineered to overexpress *Runx1* (Yanagida *et al.* 2005). Overexpression of *Runx1* caused an increased rate of leukaemia development in these mice and also caused an increase in the frequency of megakaryoblastic leukaemia (Yanagida *et al.* 2005). Together these studies suggest that the amplification of *RUNX1* is leukaemogenic in haematopoietic lineages and therefore could contribute to the development of leukaemia in individuals with aberrant overexpression of *RUNX1*.

1.2.3.4 RUNX1 Disruption and Predisposition to Leukaemia

Although chromosomal translocations, mutations and gene amplification of *RUNX1* are present in individuals with leukaemia and these alterations to *RUNX1* have been shown to contribute to leukaemogenesis, alteration to the RUNX1 gene alone is not sufficient to cause leukaemia. In keeping with the multi-hit model of leukaemogenesis as described in *Section 1.2.3*, individuals with AML which possess RUNX1 mutations have a higher frequency of mutations to signalling transduction genes such as *FLT3*, *N-RAS*, *PTPN11*

and *NF1* (Niimi *et al.* 2006, Tang *et al.* 2009). Further, this is supported by studies of transgenic RUNX1-ETO mice which do not spontaneously develop leukaemia (De Guzman *et al.* 2002, Higuchi *et al.* 2002, Rhoades *et al.* 2000, Yuan *et al.* 2001). Although these mice displayed haematopoietic developmental abnormalities which are also observed in humans with the t(8;21) chromosomal translocation, the chromosomal translocation alone was not sufficient to cause leukaemia in these mice (De Guzman *et al.* 2002, Higuchi *et al.* 2002, Rhoades *et al.* 2000, Yuan *et al.* 2001). However, treatment of these transgenic RUNX1-ETO mice with a DNA alkylating agent, N-ethyl-N-nitrosourea, resulted in a large proportion of the mice developing leukaemia; suggesting that additional mutations which cooperate with RUNX1-ETO are required for the development of leukaemia (Higuchi *et al.* 2002, Yuan *et al.* 2001). Interestingly, a further study demonstrated that mutation to the receptor kinase gene *c-Kit* in these transgenic RUNX1-ETO mice also caused the mice to develop leukaemia (Nick *et al.* 2012).

Although it has been shown that additional mutations of other genes are required to cause leukaemia, alteration to RUNX1 plays a key role in leukaemia development. Disruption to RUNX1 impacts cell differentiation, proliferation and apoptosis (Klampfer *et al.* 1996, Matsushita *et al.* 1999, Mulloy *et al.* 2002, Okuda *et al.* 1998, Westendorf *et al.* 1998) due to altered regulation of its target genes.

1.3 Regulation of Gene Expression by RUNX1

Transcription factors play a critical role in determining the gene expression profiles of a cell, which specifies their phenotype and function. They regulate transcription by binding to specific DNA sequences in regulatory regions of the genome, including proximal promoter and distal regulatory regions, to control transcription of their target genes (Latchman 1993). However, transcription factors operate within a complex layer of regulatory mechanisms, which control gene expression programs. Transcription factors generally exert their influence on transcription as part of large transcriptional complexes, which consist of other DNA bound transcription factors, transcriptional co-activators and co-repressors, and the transcriptional machinery (Kadonaga 2012). These transcriptional complexes not only interact with the DNA itself, but also with the chromatin proteins associated with the DNA. The interplay between transcription factors and the chromatin environment is complex; while transcription factors can modulate the chromatin

structure, the chromatin structure also regulates the ability of transcription factors to assemble on the DNA and regulate gene expression.

1.3.1 Chromatin Structure

In eukaryotic cells, DNA is packaged into chromatin which provides a mechanism to store the large amount of genomic material in the cell nucleus, but also provides a mechanism to control transcription of genes by regulating the accessibility of DNA to the transcription machinery (Uribesalgo and Di Croce 2011). Chromatin is composed of repeating units of nucleosomes which consist of an octamer of histone proteins, H3, H4, H2A and H2B, and approximately 146 bp of DNA is wrapped twice around the histone octamer (Luger *et al.* 1997, Uribesalgo and Di Croce 2011). The nucleosomes are connected by linker DNA and linker histone H1 protein binds to the linker DNA at the entry and exit points of the nucleosome to facilitate the folding and compaction of chromatin into higher order structures (Allan *et al.* 1980, Bednar *et al.* 1998, Virani *et al.* 2012).

At the nucleosomal level, the positioning of nucleosomes along the genome can provide a mechanism for regulating gene expression. For example, nucleosome positioning surrounding the transcription start sites of genes has been found to affect RNA polymerase II binding (Schones *et al.* 2008). Furthermore, positioning of nucleosomes at gene promoters regulates the accessibility of regulatory proteins to those regions (Jiang and Pugh 2009).

Chromatin in the cell exists in two distinct states: euchromatin and heterochromatin. Euchromatin consists of loosely packed chromatin which is more accessible for transcription, whereas heterochromatin consists of tightly packed chromatin structures that are less accessible to regulatory proteins (Kouzarides 2007). In reality however, the chromatin structure exists in a continuum between these states and can be modulated by epigenetic enzymes, which modify the DNA and histone proteins, thus influencing chromatin structure and therefore gene expression. There is now a large array of enzymes known to modify DNA and histone proteins, including DNA methyltransferases (DMTs), histone acetyl transferases (HATs), histone methyltransferases (HMTs), histone deacetylases (HDACs) and histone demethylases (HDMs).

1.3.2 DNA Methylation

DNA methylation is a stable modification to the DNA which is important for mammalian development through its role in regulating gene expression and chromatin structure (Sharma *et al.* 2010). In mammalian cells, DNA methylation primarily occurs on cytosines of cytosine-guanine (CpG) pairs by the addition of a methyl group to the fifth carbon of the cytosine nucleotide by DNA methyltransferases (Mazzio and Soliman 2012, Virani *et al.* 2012). Overall, CpGs are underrepresented throughout the genome but are clustered in short regions of DNA termed CpG islands (Jones and Takai 2001). CpG islands are frequently located at the 5' end of genes, occupying approximately 60% of human gene promoters and their methylation is generally associated with gene silencing (Jones and Takai 2001, Sharma *et al.* 2010, Wang and Leung 2004).

DNA methylation at promoter regions, is well characterised in the literature and is most commonly reported to inhibit transcription by either physically blocking transcription machinery from binding or by the recruitment of transcriptional repressors or chromatin modifying enzymes (Mazzio and Soliman 2012). However, it has now been recognised that the positioning of DNA methylation relative to the transcription start site may also play different roles in the regulation of gene expression. While DNA methylation at the transcription start site is associated with transcriptional repression, DNA methylation in the gene body is associated with active transcription and may stimulate transcription elongation or play a role in transcript splicing and alternative promoter usage (Bert *et al.* 2013, Jones 2012, Laurent *et al.* 2010, Maunakea *et al.* 2010, Tekpli *et al.* 2016). Additionally, DNA methylation found in repeat regions, such as centromeres, is important for maintaining chromosomal stability (Jones 2012).

In cancer, DNA methylation is highly dysregulated leading to hypermethylation of CpG islands at gene promoters, such as tumour suppressor genes, but overall the genome becomes hypomethylated, with hypomethylation observed at distal regulatory regions and repetitive elements (Ehrlich 2002, Virani *et al.* 2012). These changes in DNA methylation patterns cause altered gene expression profiles which contribute to the development and progression of cancer (Sharma *et al.* 2010). Altered DNA methylation profiles are frequently observed in leukaemia and has been found to correlate with different subtypes of the disease (Figueroa *et al.* 2010, Garcia-Manero *et al.* 2002, Nordlund *et al.* 2012, Pei *et al.* 2012).

1.3.3 Histone Modification

The histone proteins that make up the nucleosomes are also subject to epigenetic modification. The N-terminal tails of the histone proteins extend out from the nucleosomes and are targeted by chromatin modifying enzymes such as HATs, HMTs, HDACs and HDMs (Sharma *et al.* 2010). The histone tails can undergo a variety of post-translational modifications with more than 20 different modifications reported (Zhao and Garcia 2015), the best characterised being methylation and acetylation (Kouzarides 2007, Tessarz and Kouzarides 2014). Histone modifications function as docking sites for proteins that can recognise these modifications and recruit chromatin modifiers and remodelling enzymes to either promote transcriptional activation or repression of genes by modifying the chromatin environment (Tessarz and Kouzarides 2014). Histone modifications are also important for regulating DNA replication and DNA damage repair (Van Attikum and Gasser 2009).

Histone acetylation occurs on lysine residues and is generally associated with transcriptional activation (Virani *et al.* 2012). Histone acetylation is thought to enhance transcription through disruption of the interaction of the negatively charged DNA with the positively charged histones by neutralising the charge on the histone proteins (Virani *et al.* 2012). In addition, particular acetyl residues can act as recruitment sites for chromatin modifiers or remodelers. Maintenance of histone acetylation is controlled by HATs, which acetylate the histone proteins, and HDACs which remove acetyl groups (Virani *et al.* 2012).

Unlike histone acetylation, histone methylation can be associated with both transcriptional activation and repression (Virani *et al.* 2012). Histone methylation occurs at arginine and lysine residues of histone proteins, particularly H3 and H4 (Virani *et al.* 2012). Di-methylation of H3 lysine 9 (H3K9me2) and tri-methylation of H3 lysine 27 (H3K27me3) is associated with gene silencing, while mono-, di- and tri-methylation of histone H3 lysine 4 (H3K4me1, H3K4me2 and H3K4me3) are associated with gene expression (Pekowska *et al.* 2011). Histone methylation is maintained by HMTs, which methylate the histones, and HDMs, which remove the methyl groups (Virani *et al.* 2012).

Interestingly, particular histone methylation and acetylation marks have been mapped to promoter and enhancer regions of the genome and further have been correlated with the

activity of these regulatory elements (Creyghton *et al.* 2010, Heintzman *et al.* 2007, Liang *et al.* 2004, Santos-Rosa *et al.* 2002). A study by Heintzman *et al*. (2007) determined the histone modification profiles in IFNγ-treated and untreated cells within a 30 Mb region of the genome. It was found that active promoters possess H3K4me3, while enhancers only possessed H3K4me1 and no trimethylation (Heintzman *et al.* 2007). This is supported by other studies which have also found the H3K4me3 mark at active genes (Liang *et al.* 2004, Santos-Rosa *et al.* 2002). H3K4me2 occurs at both active and inactive genes, however H3K4me3 only occurs at active genes and the conversion of H3K4me2 to H3K4me3 is associated with gene activation (Santos-Rosa *et al.* 2002). Furthermore, methylated H3K4 as well as acetylated histone H3 lysine 9 and lysine 14 (H3K9/14) is located at the 5' regions of transcriptionally active genes (Liang *et al.* 2004). Additionally, the histone mark histone H3 lysine 27 acetylation (H3K27ac) as well as H3K4me1 is associated with active enhancers while H3K4me1 alone is associated with inactive/poised enhancers (Creyghton *et al.* 2010).

Similar to DNA methylation, histone modifications are also dysregulated in cancer. Altered histone modification profiles have been observed in leukaemia and have been shown to be able to predict event-free survival in individuals with AML (Dorrance *et al.* 2006, Gelmetti *et al.* 1998, Krivtsov and Armstrong 2007, Müller-Tidow *et al.* 2010).

1.3.4 RUNX1 Binds to Promoters/Enhancers to Regulate Gene Expression

The RUNX1 transcription factor is commonly described as a sequence-specific DNA binding protein that binds to the consensus motif TGT/cGGT through its DNA binding Runt homology domain (Meyers *et al.* 1993). The Runt homology domain is also important for the interaction with CBFβ, which is required for efficient DNA binding of RUNX1 (Gu *et al.* 2000, Kanno *et al.* 1998). The C-terminal end of the RUNX1 protein is auto-inhibitory and inhibits DNA binding, however binding of CBFβ to RUNX1 relieves this inhibition, allowing RUNX1 to bind to DNA, and exposes the transcriptional activation and inhibition domains at the C-terminal of RUNX1 (Gu *et al.* 2000, Kanno *et al.* 1998).

Early studies characterising RUNX1 demonstrated regulation of genes important for haematopoietic function. A study by Takahashi *et al*. (1995) discovered that the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene promoter possesses

the consensus binding motif for RUNX1. RUNX1 function at the promoter was determined using reporter assays and RUNX1 was found to increase GM-CSF promoter activity (Takahashi *et al.* 1995). Additionally, RUNX1 also targets the macrophage colony-stimulating factor (M-CSF) gene promoter via the RUNX1 consensus binding motif (Zhang *et al.* 1994). Gel shift assays demonstrated specific binding of RUNX1 to the consensus sequence and mutation to the sequence resulted in reduced RUNX1 binding (Zhang *et al.* 1994).

RUNX1 binding to enhancer elements has been demonstrated for a number of genes. As well as regulating the GM-CSF promoter, RUNX1 also targets the GM-CSF enhancer. RUNX1 can target two overlapping RUNX1 consensus sites within a palindromic sequence of the GM-CSF enhancer and simultaneous binding of two RUNX1 proteins to both of these consensus sites leads to activation of the enhancer (Bowers *et al.* 2010). Furthermore, RUNX1 has been shown to be important for the assembly and function of the TCRδ enhancer, enabling c-Myb enhancer binding and activation of the gene promoter (Hernández-Munain and Krangel 2002). RUNX1 targets the consensus site TGTGGT in the TCRδ enhancer and binding of RUNX1 to the enhancer leads to a conformational change to the enhancer region, allowing c-Myb to bind, resulting in transcriptional activation (Hernandez-Munain and Krangel 1994, Hernández-Munain and Krangel 2002).

1.3.5 RUNX1 Cooperates with Other Transcriptions Factors

RUNX1 commonly functions as part of large transcriptional complexes to regulate gene expression. RUNX1 can physically interact with CCAAT enhancer-binding protein (C/EBP) and function cooperatively with C/EBP to activate the MCSF receptor promoter (Zhang *et al.* 1996). RUNX1 can also interact with ETS transcription factors such as ETS-1, MEF and FLI1 (Giese *et al.* 1995, Gu *et al.* 2000, Huang *et al.* 2009, Mao *et al.* 1999). RUNX1 physically interacts with ETS-1 through the Runt homology domain and binding of ETS-1 to RUNX1 increases DNA binding of RUNX1 to a consensus site in electrophoretic mobility shift assays by 7 to 10 fold (Gu *et al.* 2000). Furthermore, RUNX1 has been shown to function cooperatively with ETS-1 to bind adjacent sites in the TCRα enhancer (Giese *et al.* 1995). RUNX1 also physically interacts with the MEF transcription factor through its Runt homology domain, and RUNX1 and MEF can function synergistically to transactivate the interleukin 3 promoter (Mao *et al.* 1999).

Additionally, RUNX1 can interact with the FLI1 transcription factor through protein-protein interactions and through these interactions, synergistically activate the c-mpl promoter which contain both RUNX and FLI1 binding motifs (Huang *et al.* 2009). Furthermore, RUNX1 physically interacts with GATA1 and transfection studies have shown that RUNX1 increases GATA1 activation of promoters with GATA1 binding sites (Elagib *et al.* 2003, Waltzer *et al.* 2003).

Interestingly, in genome-wide studies RUNX1 has been shown to frequently interact with numerous transcription factors simultaneously to form a protein complex to regulate gene expression (Beck *et al.* 2013, Tijssen *et al.* 2011, Wilson *et al.* 2010). A study by Wilson *et al*. (2010) reported simultaneous binding of six key haematopoietic transcription factors with RUNX1 in 1015 regions across the genome in haematopoietic cells. The transcription factors were bound within 200 bp or less and consensus binding motifs for all seven transcription factors were present at all regions (Wilson *et al.* 2010). Binding of all seven transcription factors was later shown to be the most common binding pattern and is associated with differential expression of genes in haematopoietic stem/progenitor cells (Beck *et al.* 2013).

1.3.6 RUNX1 Interacts with Epigenetic Modifiers

RUNX1 has also been shown to influence transcription of target genes through interactions with histone modifying enzymes such as HATs and HDACs, which alter the epigenetic state of target genes. To activate gene expression, RUNX1 interacts with the histone acetyltransferases p300 and CBP (Kitabayashi *et al.* 1998, Oakford *et al.* 2010). A study by Kitabayashi *et al.* (1998) used immunoprecipitation analysis to show that RUNX1 interacts with the histone acetyltransferase p300 and CBP. The C-terminal region of RUNX1 was required for the interaction with p300 and cooperation of RUNX1 and p300 is essential for RUNX1 activation of the MPO promoter, as RUNX1 or p300 alone did not transactivate the promoter (Kitabayashi *et al.* 1998). Another study analysing RUNX1 regulation of the GM-CSF gene found that RUNX1 is required for the hyperacetylation of the GM-CSF promoter via the interaction with CBP (Oakford *et al.* 2010). CBP was found to co-activate the GM-CSF promoter and the presence of CBP at the promoter was associated with RUNX1 binding and histone hyperacetylation (Oakford *et al.* 2010). It was suggested that CBP is both required for the acetylation of chromatin

at the promoter and the acetylation of RUNX1 which increases transcriptional activity of the transcription factor (Oakford *et al.* 2010).

While RUNX1 has most commonly been described as a transcriptional activator, it can also repress gene expression. This has been shown to be dependent on the interaction with the mSin3A co-repressor, which forms a repressor complex with HDACs, and SUV39H1 histone methyltransferase (Lutterbach *et al.* 2000, Reed-Inderbitzin *et al.* 2006). A study by Lutterbach *et al*. (2000) demonstrated using immunoprecipitation assays that RUNX1 interacts with the co-repressor mSin3A and RUNX1 repression of the $p21^{Waf1/Cip1}$ promoter is dependent on this interaction. A RUNX1 deletion mutant, which could not interact with mSin3A, failed to repress $p21^{Waf1/Cip1}$ transcription (Lutterbach *et al.* 2000). Furthermore, RUNX1 has been shown to play a role in gene repression and possibly gene silencing during cell development via the interaction with repressive histone and DNA modifiers. To repress and silence the CD4 gene during T-cell maturation, RUNX1 interacts with HDACs as well as the histone methyltransferase SUV39H1, which is involved in gene silencing (Reed-Inderbitzin *et al.* 2006).

1.3.7 Involvement of RUNX1 in Higher Order Chromatin Structures

RUNX1 is also involved in the formation of higher order chromatin structures to promote the expression of genes important in haematopoiesis. RUNX1 has been shown to be important for some enhancer-promoter interactions. A study by Levantini *et al*. (2011) found that RUNX1 regulates the CD34 gene through an enhancer located 17.4-19.6 kb downstream of the transcription start site, which is required for *CD34* expression. It was discovered that the downstream enhancer region physically interacts with the CD34 promoter and targeted mutagenesis of RUNX1 consensus sites leads to disruption of this interaction and decreased *CD34* expression in haematopoietic cells (Levantini *et al.* 2011). RUNX1 is therefore required to facilitate the interaction between the downstream CD34 enhancer and CD34 promoter, to enable *CD34* expression in haematopoietic cells.

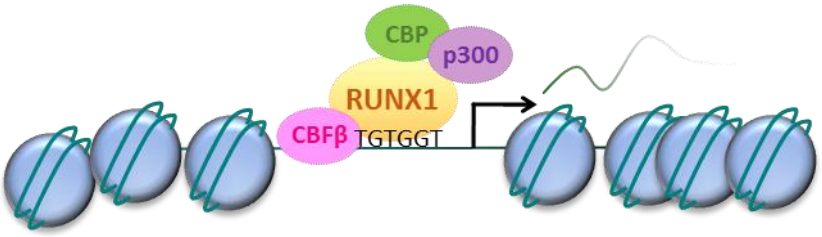
Additionally, RUNX1 has been implicated in chromatin unfolding of transcription factor genes vital for haematopoiesis during development. A study by Hoogenkamp *et al*. (2009) demonstrated that transient binding of RUNX1 to *PU.1* regulatory elements is required for the early chromatin unfolding and expression of the PU.1 gene important for myelopoiesis. Hoogenkamp *et al.* (2009) suggest that once the haematopoietic

transcription factors are expressed during development, stable transcription factor complexes are formed on the genes and active chromatin is maintained without RUNX1 (Hoogenkamp *et al.* 2009).

1.3.8 Disruption of RUNX1 in Leukaemia

Not surprisingly, disruption to RUNX1 leads to genome-wide changes in gene expression due to altered regulation of its target genes. For example, the RUNX1-ETO fusion protein, which is produced from the t(8;21) chromosomal translocation, can bind to RUNX1 target genes since the RUNX1 DNA binding domain is retained, however, its effect on gene expression is different to RUNX1 due to the presence of the ETO protein. As demonstrated in *Figure 1.3*, RUNX1 normally interacts with transcriptional co-activators such as p300 and CBP to activate gene expression (Kitabayashi *et al.* 1998), however, due to the replacement of the transactivation domain of RUNX1 with ETO, expression of target genes is generally repressed due to interactions with repressive co-factors such as N-CoR, mSin3A and HDACs (Lutterbach *et al.* 1998, Wang *et al.* 1998).

A



B

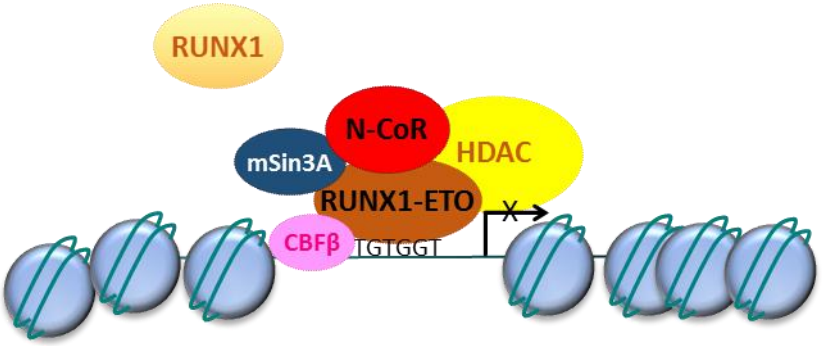


Figure 1.3 – RUNX1 and RUNX1-ETO effects on transcription. Schematic of **A)** RUNX1 and **B)** RUNX1-ETO regulation of RUNX1 target genes. RUNX1 and RUNX1-ETO both bind to RUNX1 consensus sites at target genes and heterodimerise with CBFβ. RUNX1 interacts with histone acetyltransferases CBP and p300 to activate gene expression, while RUNX1-ETO blocks RUNX1 binding and represses gene expression by interacting with co-repressors mSin3A, N-CoR and HDACs. DNA is shown as aqua lines wrapped around nucleosomes (blue balls). Transcription start site is shown by the black right-angled arrow.

1.3.9 Use of Genome-Wide Studies to Understand RUNX1 Function

Although many target genes of RUNX1 have been identified through candidate gene analysis, the full repertoire of RUNX1 controlled genes remains to be determined. More recently, the advent of genome-wide technology has facilitated the understanding of the spectrum of genes under the control of RUNX1.

In the 2000's, microarray studies by Ichikawa *et al*. (2006), Michaud *et al.* (2008), Valk *et al.* (2004) and Wotten *et al.* (2008) aimed to identify gene expression profiles of haematopoietic cells with RUNX1 or CBFβ disrupted. Through these studies, genes involved in several cellular pathways were identified as potential RUNX1 target genes. Interestingly, genes which play an important role in the cell cycle, cell growth and proliferation, and DNA replication and repair had altered expression in haematopoietic cells with RUNX1 or CBFβ disrupted, suggesting that RUNX1 may be important in regulating these pathways (Ichikawa *et al.* 2006, Michaud *et al.* 2008, Valk *et al.* 2004, Wotton *et al.* 2008). Disruption to these pathways are known to play key roles in cancer development (Evan and Vousden 2001, Molinari 2000) and it has been demonstrated that disruption to RUNX1 leads to increased cell proliferation (Matsushita *et al.* 1995, Mulloy *et al.* 2002, Okuda *et al*. 1998, Rhoades *et al.* 2000), inhibition of cell differentiation (Burel *et al.* 2001, Heidenreich *et al.* 2003, Tonks *et al.* 2004, Westendorf *et al.* 1998) and inhibition of apoptosis (Klampfer *et al.* 1996, Matsushita *et al.* 1999).

Interestingly, genes involved in cell adhesion and migration were also found to have altered expression in haematopoietic cells when RUNX1 or CBFβ gene expression was disrupted, suggesting that RUNX1 may also regulate genes involved in these pathways (Ichikawa *et al.* 2006, Michaud *et al.* 2008, Valk *et al.* 2004, Wotton *et al.* 2008). While genes involved in cell adhesion and migration pathways have been linked to cancer cell metastasis (Albelda 1993, Hood and Cheresh 2002), the role of RUNX1 in cell adhesion and migration in haematopoietic cells has not been explored. Many of these genes identified to be involved in cell adhesion and migration were found to encode cell surface or extracellular ligands (Ichikawa *et al.* 2006, Michaud *et al.* 2008, Valk *et al.* 2004, Wotton *et al.* 2008). Expression of two genes encoding integrin adhesion receptors, ITGB4 and ITGB5, were commonly identified to be disrupted in these microarray studies, therefore suggesting that they may be regulated by RUNX1 (Ichikawa *et al.* 2006, Michaud *et al.* 2008, Valk *et al.* 2004, Wotton *et al.* 2008).

In addition, previous microarray studies conducted in our laboratory have also identified the integrin genes, ITGB4 and ITGB5, as potential targets of RUNX1 (Oakford and Holloway, unpublished). *RUNX1* expression was knocked down in Jurkat T cells by transfecting the cells with siRNAs targeted to *RUNX1*. It was found that the decrease in *RUNX1* expression caused an increase in *ITGB4* and *ITGB5* expression.

1.4 RUNX1 and Integrins

1.4.1 Integrins

Integrins are transmembrane adhesion receptors which are involved in cell-ECM and cell-cell interactions (Barczyk *et al.* 2010, Takada *et al.* 2007). Integrin receptors are heterodimeric and consist of non-covalently bound alpha and beta glycoprotein subunits (van der Flier and Sonnenberg 2001). Integrins are comprised of three domains: an extracellular ligand-binding globular head, a hydrophobic transmembrane domain and an intracellular cytoplasmic tail (Scales and Parsons 2011). The integrins form a large family and in humans at least 18 α and 8 β subunits have been identified, forming at least 24 heterodimeric receptors (Figure 1.4) (Shimaoka and Springer 2003). Integrins function in linking the exterior of the cells to the interior by connecting the ECM or other cells to the cytoskeleton (Margadant *et al.* 2011, Takada *et al.* 2007, van der Flier and Sonnenberg 2001). Integrins bind to specific ligands and therefore can be grouped based on their ligand specificity, which is shown in *Figure 1.4*. In humans, there are laminin-binding integrins, collagen-binding integrins, leukocyte-specific integrins and arginylglycylaspartic acid (RGD)-recognising integrins (Barczyk *et al.* 2010, Takada *et al.* 2007).

Although integrins play an important role in maintaining tissue integrity through adhesive interactions between other cells and the ECM, they also play a vital role as signalling receptors (Ivaska and Heino 2010). Integrins can transmit bi-directional signals across the plasma membrane through inside-out and outside-in signalling (Hu and Luo 2013). Inside-out signalling generally causes the activation of integrins in the low-affinity state, through signalling from within the cell, which results in the conformational change of the integrins into a high affinity state (Hu and Luo 2013). Once integrins are activated and have a high affinity for ligands, binding of extracellular ligands to integrins then transmit

signals into the cell through outside-in signalling (Hu and Luo 2013). Outside-in signalling can have a significant effect on the cell and it has been found to modulate cell adhesion, proliferation, survival, shape, polarity, motility, differentiation and gene expression (Hu and Luo 2013, Takada *et al.* 2007). Integrins play important roles in many biological pathways such as development, angiogenesis, inflammation, tissue repair and also homeostasis of the haematopoietic system (Shimaoka and Springer 2003).

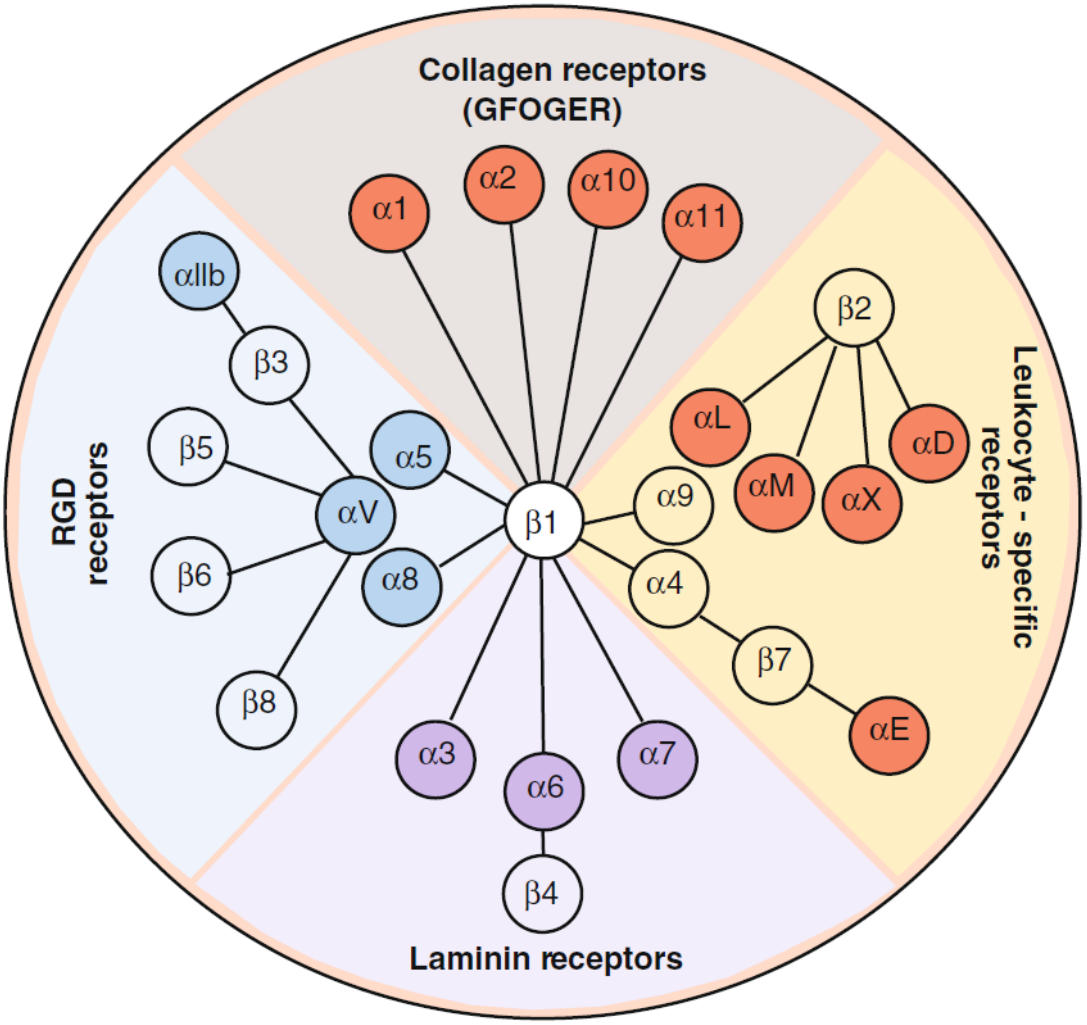


Figure 1.4 – The integrin family. This diagram represents the various α and β subunits produced by specific integrin genes in humans. The solid lines joining the different subunits depict the formation of a heterodimer and the different type of heterodimeric integrin receptors are shown. Taken from Barczyk *et al.* (2010).

1.4.2 The Role of Integrins in Haematopoiesis

The interactions of integrins on haematopoietic progenitors and stem cells with bone marrow ligands is important for the regulation of haematopoiesis (Hurley *et al.* 1995, Prosper and Verfaillie 2001). In the bone marrow, haematopoietic progenitors grow in close proximity to stromal cells which produce regulatory cytokines that have a positive and negative effect on the proliferation and differentiation of the haematopoietic progenitors (Eaves *et al.* 1991, Hurley *et al.* 1995, Watowich *et al.* 1996). Integrins on haematopoietic cells can bind to ligands on stromal cells as well as the stromal ECM and these interactions can also have an effect on the proliferation and differentiation of haematopoietic cells (Verfaillie *et al.* 1994).

In early research into the role of integrins in haematopoiesis, it was found that proliferation of haematopoietic cells was significantly higher when the cells were cultured away from stromal cells compared to cells which were adherent to the stroma, suggesting that close stroma and haematopoietic progenitor cell interactions could function in regulating or inhibiting haematopoietic cell proliferation (Hurley *et al.* 1995, Verfaillie and Catanzaro 1996). It was also found that the integrin receptor α4β1, also known as very late activation antigen-4 (VLA-4), is responsible for the adhesion of haematopoietic cells to the stroma, and thus could play a role in the regulation of haematopoietic proliferation (Hurley *et al.* 1995). Further studies revealed that adhesion of haematopoietic cells to the bone marrow ECM component fibronectin via α4β1 receptors is important for the adhesion and retention of haematopoietic cells in the bone marrow, as well as the regulation of proliferation and differentiation of haematopoietic cells (Hurley *et al.* 1995, Krämer *et al.* 1999, Schofield *et al.* 1998, Verfaillie *et al.* 1991, Weinstein *et al.* 1989, Williams *et al.* 1991).

As well as the proliferation and differentiation of haematopoietic cells, integrins have been found to play a role in the homing and mobilisation of haematopoietic cells in the bone marrow. During steady-state haematopoiesis, there is a continuous mobilisation of haematopoietic stem cells into the peripheral blood and homing of haematopoietic stem cells from the peripheral blood back into the bone marrow (Kondo *et al.* 2003, Qian *et al.* 2006). The role of the continuous mobilisation and homing of haematopoietic stem cells is not well understood, but it is a physiological process which occurs in adults and is utilised in stem cell transplantation for individuals with leukaemia (Bonig and

Papayannopoulou 2012, Kondo *et al.* 2003). It is thought that the constant mobilisation and homing of haematopoietic stem cells in the adult is important for sustained normal haematopoiesis (Papayannopoulou and Nakamoto 1993).

The integrin receptor α4β1 has been found to play a central role in mobilisation and homing of haematopoietic cells. Deletion or inhibition of α4 integrin results in the mobilisation and accumulation of haematopoietic stem cells in the blood stream (Papayannopoulou and Nakamoto 1993, Scott *et al.* 2003). Furthermore, in transplantation studies in mice, the conditional deletion of α4 integrin impairs the homing of haematopoietic stem cells to the bone marrow (Scott *et al.* 2003). Studies have shown that the expression of the α4 integrin and the activation state of the α4β1 integrin receptor is correlated with the mobilisation and homing of haematopoietic stem cells to the bone marrow. Haematopoietic stem cells in the peripheral blood have decreased expression of α4 and the α4β1 receptor is deactivated, whereas haematopoietic stem cells in the bone marrow have increased expression of α4 and the α4β1 receptor is in an activated state (Lichterfeld *et al.* 2000, Prosper *et al.* 1998). Similar results have been reported for the β1 integrin. In transplantation studies, β1 null haematopoietic stem cells failed to engraft in irradiated recipient mice due to impaired homing of the haematopoietic stem cells to the bone marrow (Potocnik *et al.* 2000), suggesting both α4 and β1 are important for mobilisation and homing of haematopoietic stem cells.

In addition to the α4β1 receptor, the α5β1 receptor, also known as VLA-5, is also expressed on haematopoietic cells and plays a role in the homing and mobilisation of haematopoietic cells in the bone marrow (Levesque *et al.* 1995, Van der Loo *et al.* 1998, Wierenga *et al.* 2006). Additionally, the α6β1 receptor, which is expressed in human and mouse haematopoietic stem and progenitor cells, is involved in homing of foetal liver multi-lineage repopulating haematopoietic stem cells in mice (Qian *et al.* 2006). Inhibition of the α6 integrin by α6 antibodies was found to inhibit stem cell homing, but did not have an effect on the mobilisation of stem cells in the bone marrow (Qian *et al.* 2006). Further studies of α6 integrin in the bone marrow determined that α6 is not required for the homing and engraftment of multi-lineage repopulating haematopoietic stem cells and is only functional during the homing of foetal liver haematopoietic stem cells (Qian *et al.* 2007).

1.4.3 Involvement of Integrins in Leukaemia

Not surprisingly, given their physiological roles, integrins also play key roles in cancer development and progression. Integrins have been implicated in cancer cell proliferation, invasion, migration and survival (Desgrosellier and Cheresh 2010, Guo and Giancotti 2004, Hood and Cheresh 2002). The up-regulation and down-regulation of integrins has been linked to many cancers including leukaemia, melanoma, glioblastoma, non-small-cell lung carcinoma and breast, colon, kidney, lung, ovarian, cervical, pancreatic, prostate and skin cancer (Desgrosellier and Cheresh 2010, Mizejewski 1999). Tumour cells increase expression of integrins that aid in proliferation, survival and migration, whereas integrins that inhibit cancer growth, survival and migration are down-regulated (Guo and Giancotti 2004). Integrin expression on cancer-associated cells can also contribute to the development of cancer by facilitating angiogenesis, desmoplasia, lymphangiogenesis and inflammation (Desgrosellier and Cheresh 2010).

As mentioned previously, integrins regulate haematopoiesis through promoting the interaction of haematopoietic cells with bone marrow stromal cells, as well as the stromal ECM. Disruption of these interactions necessarily disturbs haematopoietic processes. Not surprisingly then, the dysregulation and disruption to integrin genes has been identified in many types of leukaemia including acute myeloid leukaemia (Brouwer *et al.* 2001), B cell chronic lymphocytic leukaemia (CLL) (Csanaky *et al.* 1997), B cell acute lymphoblastic leukaemia (Geijtenbeek *et al.* 1999) and chronic myeloid leukaemia (CML) (Verfaillie *et al.* 1992). Although the involvement of integrin genes in leukaemia was identified more than 20 years ago, the importance of integrins in leukaemia development, therapy resistance and potential biomarkers/therapeutic targets for the disease have only been elucidated more recently.

In CML, the haematopoietic cells are released prematurely from the bone marrow and proliferate aberrantly due to decreased adhesion to the bone marrow stroma and fibronectin through β1 heterodimeric receptors (Bhatia *et al.* 1996, Verfaillie *et al.* 1992). In normal haematopoietic development, engagement of β1 receptors on haematopoietic stem cells with fibronectin within the bone marrow causes decreased cell proliferation due to β1-mediated inhibitory signalling (Hurley *et al.* 1995). CML cells also express the integrins α2 and α6 which bind to laminin and collagen, whereas the expression of these integrins are usually absent from normal haematopoietic cells (Verfaillie *et al.* 1992). It

is therefore suggested that aberrant proliferation of CML haematopoietic cells could be due to a combination of a lack of inhibitory cell proliferation signals due to decreased adhesion of haematopoietic cells to stroma and fibronectin through β1 receptors, and a gain of adhesive interactions with laminin and collagen due to an up-regulation of α2 and α6 integrins, therefore causing an early release of haematopoietic cells from the bone marrow stroma.

In addition to altered adhesion of haematopoietic cells in CML, altered adhesion of haematopoietic cells has also been observed in B-lineage ALL. Similar to the decrease in adhesion through β1 receptors observed in CML (Bhatia *et al.* 1996, Verfaillie *et al.* 1992), it has also been shown that individuals with ALL have decreased adhesion through α4β1 integrin receptor (Geijtenbeek *et al.* 1999). It was found that 7 out of 20 individuals with ALL possessed leukaemic cells which expressed normal levels of the α4β1 integrin receptor, however the receptor was not functional in those cells, suggesting a defect in β1 integrin signalling (Geijtenbeek *et al.* 1999). In addition to altered function of α4β1, high levels of the integrin receptor have also been observed in children with B-cell precursor ALL at first relapse and is associated with poor outcome (Shalapour *et al.* 2011). It was discovered that high expression of α4β1 at first relapse was associated with poor prognosis, poor response to therapy and decreased likelihood of event-free and overall survival (Shalapour *et al.* 2011).

In addition to CML and ALL, altered expression of the α4β1 integrin receptor has also been associated with CLL (Baldini *et al.* 1992, Eksioglu-Demiralp *et al.* 1996). In an early study it was shown the α4β1 receptor was expressed at higher levels in 37.1% of individuals with B-cell CLL compared to normal B cells (Baldini *et al.* 1992). In addition, the study also reported that the α3β1 integrin receptor was expressed at higher levels in 87.1% of individuals with CLL compared to normal B cells (Baldini *et al.* 1992). Furthermore, another study conducted a few years later, discovered that in B-cell CLL, expression of the α4 subunit is related to different stages of the disease (Eksioglu-Demiralp *et al.* 1996). Individuals in the early stage of CLL were shown to have lower expression of α4 compared to normal controls, whereas individuals in the advanced stage of CLL were shown to have higher expression of α4 compared to normal controls (Eksioglu-Demiralp *et al.* 1996). In support of these findings, recent studies have shown that high expression of α4 in individuals with CLL is associated with advanced clinical

stage of the disease as well as aggressive disease biology (Baumann *et al.* 2016) and also is associated with poor overall survival (Dal Bo *et al.* 2016).

Changes in α4β1 expression has also been reported in AML, however the studies have reported conflicting results. In earlier studies it was found that individuals with AML positive for α4β1 had significantly lower complete remission rates, higher relapse rates and lower overall survival rates at 5 years (Matsunaga *et al.* 2003). However, in contrast to these findings, more recent studies reported that in paediatric AML, individuals with high α4β1 expression were found to have a lower relapse rate and higher disease-free survival after 3 years compared to individuals with low α4β1 expression (Walter *et al.* 2010). Furthermore, in newly diagnosed adult individuals with AML, individuals with favourable or intermediate cytogenetic risk were found to have higher levels of α4β1 expression (Bae *et al.* 2015). In addition, individuals with higher levels of α4β1 had a higher probability of complete remission, showed longer relapse-free survival and higher overall survival rates when compared to individuals with lower levels of α4β1 (Bae *et al.* 2015). The differences in the outcomes associated with α4β1 expression may be due to other genetic abnormalities present in these individuals contributing to the different disease states.

In addition to the α4β1 receptor, increased expression of integrin genes ITGAM, ITGAL, ITGB4 and ITGA6 have also been associated with AML, with increased expression of these integrins associated with poorer outcomes (Brouwer *et al.* 2001, Chen *et al.* 2013, Paietta *et al.* 1998, Xu *et al.* 2015, Yamakawa *et al.* 2012). Increased levels of ITGAM integrin gene has been associated with lower complete remission rate and shorter overall survival (Chen *et al.* 2013, Paietta *et al.* 1998, Xu *et al.* 2015). Additionally, the ITGAL integrin gene has been associated with a significantly shorter five-year survival in patients with high expression of the integrin gene (Brouwer *et al.* 2001). Furthermore, ITGB4 and ITGA6 integrin genes, which form the α6β4 integrin receptor, has been associated with increased drug resistance in AML with high EVI1 expression (Yamakawa *et al.* 2012).

1.4.3.1 Involvement of Integrins in Drug Resistance in Leukaemia

The importance of integrins in drug resistance has only been highlighted in recent years. In AML, approximately 60-70% of patients will relapse due to minimal residual disease (Venditti *et al.* 2000). AML cells have been shown to bind to bone marrow ECM

components fibronectin and laminin through α4β1 and α5β1 integrin receptors (Bendall *et al.* 1993). It has also been shown that AML cells bind to bone marrow stromal cells through a combination of β1 and β2 integrin mechanisms, and this is also true for B-CLL cells (Bendall *et al.* 1993, Lagneaux *et al.* 1999). In a more recent study, the α4β1 receptor was shown to contribute to drug resistance through the interaction with fibronectin expressed by bone marrow stromal cells (Matsunaga *et al.* 2003). Interaction of α4β1 on AML cells with fibronectin activated the PI-3K/AKT/Bcl-2 signalling pathway, therefore resulting in resistance to drug-induced apoptosis (Matsunaga *et al.* 2003). More recently it was found that that the inhibition of α4β1 integrin receptor by specific inhibition of the α4 integrin sensitised chemotherapy-resistant pre-B ALL cells to chemotherapy (Hsieh *et al.* 2013), further confirming the importance of α4β1 in leukaemia, particularly AML and ALL/CLL.

Minimal residual disease is also responsible for drug resistance observed in CML and is also caused by β1-integrin mediated adhesion but through α5β1 integrin receptor to fibronectin of bone marrow stromal cells (Damiano *et al.* 2001). It has been shown that the BCR-ABL fusion protein frequently observed in CML increases β1-integrin mediated adhesion to stromal cells (Fierro *et al.* 2008), and interaction of CML cells with fibronectin of stromal cells results in the cells becoming resistant to apoptosis induced by BCR-ABL inhibitors, DNA damaging agents and γ-irradiation (Damiano *et al.* 2001). The use of a chemotherapeutic drug, arsenic trioxide, on a CML cell line, K562, was shown to overcome cell adhesion-mediated drug resistance by down-regulating the expression of β1 integrin (Guo-Bao *et al.* 2010). Additionally, in ALL which is positive for the BCR-ABL fusion protein, the inhibition of α5β1 integrin, by the use of a α5 antibody, inhibited adhesion of leukaemic cells to fibronectin (Hu and Slayton 2014). The use of the α5 antibody was found to act synergistically with the chemotherapeutic drug imatinib to induce apoptosis in the leukaemic cells (Hu and Slayton 2014). It was suggested that by altering the interactions of leukaemic cells positive for BCR-ABL with the bone microenvironment, it may increase their susceptibility to therapy (Hu and Slayton 2014).

Taken together, these studies suggest that integrin receptors α4β1 and α5β1 play important roles in cell adhesion-mediated drug resistance in leukaemia. The expression and function of β1 integrin receptors, particularly α4β1, is disrupted in leukaemia, contributing to the irregular development of the haematopoietic cells, due to a change in

interactions of the haematopoietic cells with the bone marrow stroma. However, leukaemic cells have also been shown to up-regulate integrin receptors, particularly α4β1, to increase binding to bone marrow stroma components, which allows the cells to become drug resistant and is thought to contribute to minimal residual disease. In fact, it has recently been proposed that leukaemic cells may manipulate the expression of integrin genes during the course of the malignancy for their development and survival, as shown in *Figure 1.5* (Brachtl *et al.* 2014). Leukaemic cells may up-regulate α4β1 to bind to bone marrow stromal cells which increases their survival and resistance to drugs (Brachtl *et al.* 2014). The leukaemic cells may then down-regulate α4β1 expression to decrease their interaction with the bone marrow, therefore allowing the cells to proliferate and then leave the bone marrow, migrating to the blood stream (Brachtl *et al.* 2014). The leukaemic cells may then migrate back into the bone marrow by increasing α4β1 expression which allows the cells to bind to the endothelial cells of the bone marrow and enter the bone marrow through transendothelial migration (Brachtl *et al.* 2014).

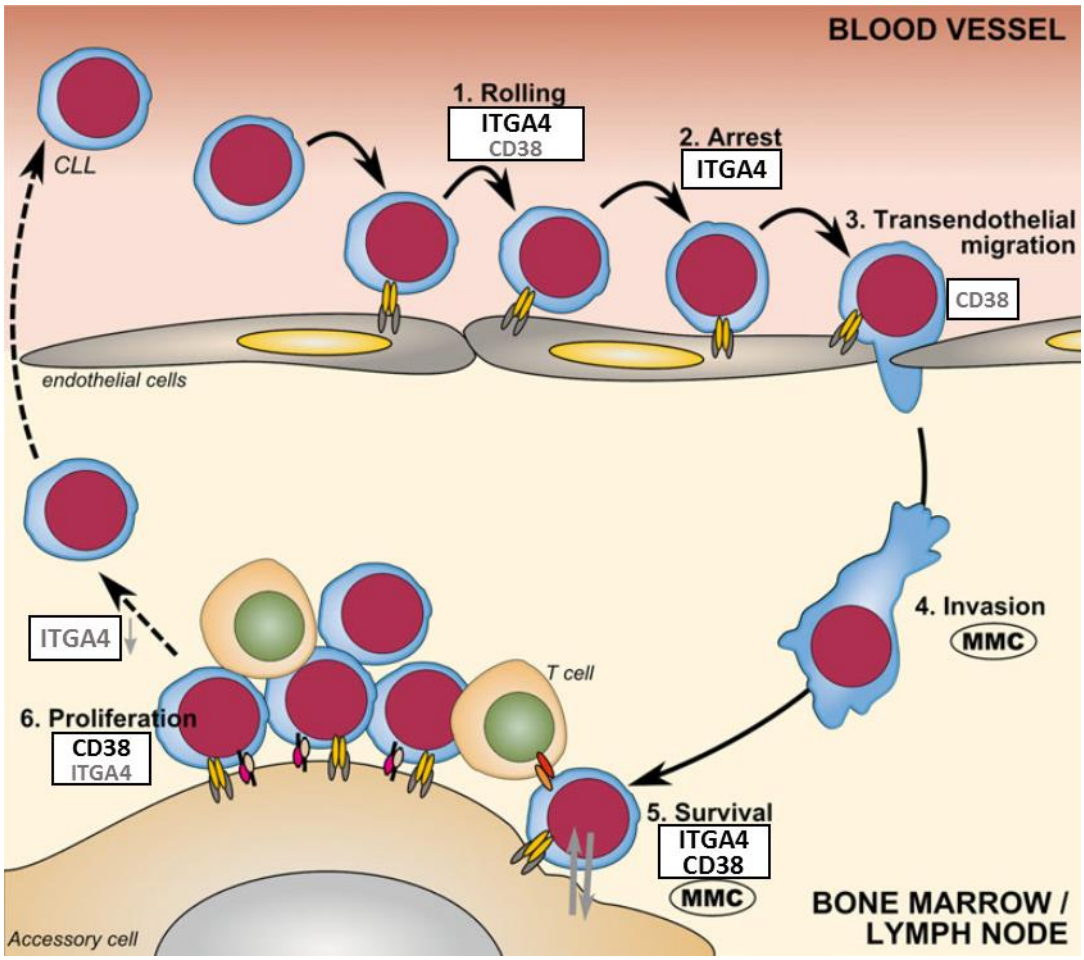


Figure 1.5 – Mobilisation and homing of leukaemic cells. Figure adapted from Brachtl *et al*. (2014) shows the proposed disease development model of CLL. CLL cells bind to endothelial cells of the bone marrow/lymph node through α4β1 integrin receptors (ITGA4 is expressed) and CLL cells roll along the endothelial cells where the cells are arrested. The CLL cells then enter the bone marrow/lymph node through transendothelial migration, which may contribute, to CD38 expression. A macromolecular complex (MMC) is important for the invasion of CLL cells within the bone marrow and lymphoid tissue. Binding of CLL cells to bone marrow or lymph node cells through α4β1 and CD38 receptors, as well as the MMC, promotes survival. Expression of CD38 promotes proliferation of CLL cells within the bone marrow, and decrease of ITGA4 expression allows the CLL cells to detach from the bone marrow/lymphoid cells and migrate to the blood stream. ITGA4 and CD38 in light grey text shows hypothesised roles of the receptors. MMC: macromolecular complex including ITGA4, ITGB1, CD38, MMP9 and CD44v.

1.4.4 Regulation of Integrin Genes by Epigenetic Mechanisms

It is known that gene expression is controlled by epigenetic and transcriptional mechanisms, however the specific mechanisms that control integrin genes are relatively uncharacterised. However, recent evidence suggests that integrin genes may be regulated by epigenetic mechanisms such as DNA methylation and histone modification. These mechanisms have also been found to be disrupted in many types of cancer.

The ITGA4 gene, which encodes the α4 subunit, is aberrantly methylated in human gastric cancer (Park *et al.* 2004). It has been reported that ITGA4 gene expression is lost in gastric cancer cell lines due to CpG methylation-dependent silencing (Park *et al.* 2004). Hypermethylation of the ITGA4 gene is also observed in early stage oesophageal squamous cell carcinoma (Lee *et al.* 2008) and prostate cancer (Mostafavi-Pour *et al.* 2015). In CLL with trisomy 12, expression of the ITGA4 gene was found to increase due to hypomethylation of the ITGA4 gene, whereas low expression of *ITGA4* in CLL without trisomy 12 was due to increased methylation of the ITGA4 gene (Zucchetto *et al.* 2013). In addition, integrin genes ITGA7 and ITGA2 have been found to be regulated by methylation. In malignant pleural mesothelioma, cells have increased migratory potential due to the down-regulation of *ITGA7* caused by hypermethylation (Laszlo *et al.* 2015). It was found when cells have forced *ITGA7* expression, cell motility was inhibited (Laszlo *et al.* 2015). In prostate cancer cell lines, expression of the ITGA2 gene was found to be correlated with methylation of key CpG sites within the ITGA2 promoter (Chin *et al.* 2015).

In addition to DNA methylation, histone modification is another epigenetic mechanism found to regulate integrin gene expression. In hepatocellular carcinoma cell line (Hep3B), treatment with a HDAC inhibitor resulted in the activation and increased expression of integrin genes ITGA4, ITGB2 and ITGB6, while resulting in a down-regulation of ITGA6, ITGA10 and ITGB8 integrin genes (Lin *et al.* 2005). Furthermore, treatment of haematopoietic stem cells and acute myeloid leukaemia blast cells with a HDAC inhibitor resulted in down-regulation of integrin receptor α4β1 (Mahlknecht and Schönbein 2008). Decreased expression of α4β1 was associated with decreased adhesion to mesenchymal stromal cells. It was therefore concluded that HDAC inhibitor treatment may inhibit stem cell homing, but it may improve peripheral blood stem cell mobilisation, which could help reduce minimal residual disease from AML (Mahlknecht and Schönbein 2008).

1.4.5 Regulation of Integrins by RUNX Transcription Factors

Through previous studies, both integrin genes and RUNX transcription factors have been found to be commonly disrupted in leukaemia (Brouwer *et al.* 2001, Csanaky *et al.* 1997, Dal Cin *et al.* 2001, Geijtenbeek *et al.* 1999, Miyoshi *et al.* 1991, Osato *et al.* 1999, Verfaillie *et al.* 1992), suggesting that both classes of proteins play important roles in leukaemogenesis. Interestingly, there is evidence to suggest that RUNX transcription factors may regulate integrin genes in haematopoietic cells. In lymphoid and myeloid cells, the ITGAL integrin gene has been found to be regulated by RUNX1 and RUNX3, respectively (Puig-Kröger *et al.* 2000, Puig-Kröger *et al.* 2003). Both RUNX1 and RUNX3 bind to the ITGAL promoter, and disruption to this site resulted in a significant decrease in promoter activity (Puig-Kröger *et al.* 2000, Puig-Kröger *et al.* 2003). When RUNX3 was overexpressed in the human lymphoid cell line, U937, the expression of *ITGAL* was also increased, and chromatin immunoprecipitation assays confirmed RUNX3 binding to the ITGAL promoter *in vivo* (Puig-Kröger *et al.* 2003). In mature monocyte-derived dendritic cells, the integrin gene ITGA4 has also been found to be targeted by the RUNX3 transcription factor (Domínguez-Soto *et al.* 2005). RUNX3 could activate the ITGA4 promoter and increased expression of *ITGA4* in mature monocyte-derived dendritic cells correlated with increased expression of *RUNX3* (Domínguez-Soto *et al.* 2005). Altogether, these studies demonstrate that RUNX transcription factors have the potential to regulate integrin genes, which suggests that the disruption to RUNX transcription factors in leukaemia could lead to altered expression of integrin genes due to altered regulation by RUNX transcription factors.

1.5 Research Aims

RUNX1 plays an important role in haematopoiesis and is one of the most commonly disrupted genes in leukaemia. Understanding the mechanism by which it regulates gene expression in key pathways, is crucial for understanding its role in leukaemia development and identifying potential therapeutic targets in the disease. Microarray studies conducted by our team, and others, have identified several integrin genes as putative novel RUNX1 target genes. As detailed above, integrins are important for haematopoiesis by facilitating interactions between haematopoietic cells and extracellular matrix components of the bone marrow and haematopoietic tissues. Integrin expression

is also altered in many leukaemias, however the regulation of integrin gene expression is poorly understood. Hence, the hypothesis of this project is: *The RUNX1 transcription factor and epigenetic mechanisms cooperatively regulate the expression programs of key integrins in haematopoietic cells and disruption of RUNX1 and epigenetic mechanisms is responsible for altered expression of these integrins in leukaemic cells.* To address this hypothesis, three main aims were addressed:

1. Determine the integrin genes that are regulated by RUNX1 in haematopoietic cells.
2. Characterise RUNX1 regulation of selected integrin genes in haematopoietic cells.
3. Determine the epigenetic mechanisms that regulate the selected integrin genes in haematopoietic cells.

Chapter 2

Materials and Methods

2.1 Cell Culture

Commercially available leukaemic cell lines were used to analyse integrin and RUNX1 expression, as well as RUNX1 and epigenetic regulation of integrin genes.

2.1.1 Cell Lines and Culture Conditions

Cells lines K562, KG-1a and Kasumi-1 were purchased from American Type Culture Collection and were cultured in Gibco® Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, USA) containing D-glucose (2 g/L), glutathione (1 mg/L), HEPES (6 g/L) and sodium bicarbonate (2 g/L) which was supplemented with 10% heat-inactivated (56 ˚C for 30-60 minutes) foetal bovine serum (FBS; 20% for Kasumi-1 cells; Life Technologies, USA) and penicillin (100 U/mL)/streptomycin (100 µg/mL, Sigma-Aldrich, USA). K562 cells were subcultured every 2-3 days and were maintained between $1x10^5$ and $1x10^6$ cells/mL; KG-1a cells were also subcultured every 2-3 days and were maintained between $2.0x10^5$ and $1.0x10^6$ cells/mL; and Kasumi-1 cells were subcultured once a week and maintained between $2.0x10^5$ and $2.0x10^6$ cells/mL. All cell lines were cultured in a humidified incubator at 37˚C and 5% CO_2.

2.1.2 Cell Treatments

2.1.2.1 Phorbol 12-myristate 13-acetate Treatment

The effect of phorbol 12-myristate 13-acetate (PMA) treatment on integrin expression was examined in leukaemic cell lines. K562 and KG-1a cells lines were subcultured to $2x10^5$ cells/mL and were treated with PMA (20 ng/mL; Boehringer Mannheim, Australia) for 72 hours. Kasumi-1 cells were subcultured to $4x10^5$ cells/mL and were treated as described for K562 and KG-1a cell lines.

2.1.2.2 5-aza-2'-deoxycytidine and Trichostatin A Treatment

To determine if integrin gene expression is affected by the DNA methylation and histone acetylation status of the gene, leukaemic cell lines were treated with epigenetic inhibitors, 5-aza-2'-deoxycytidine (DAC) and Trichostatin A (TSA).

DAC, which is currently being used as a therapy for AML (Blum *et al.* 2010, Cashen *et al.* 2010, Issa *et al.* 2004), is a potent DNA methylation inhibitor. Once taken up by cells, DAC is metabolised to form DAC-triphosphate which can be used as substrate for the DNA replication machinery, and therefore can be incorporated into the DNA, replacing normal cytosines (Stresemann and Lyko 2008). Azacytosine-guanine dinucleotides are recognised by DNA methyltransferases which bind to the azacytosine via a covalent bond (Stresemann and Lyko 2008). While this covalent bond is normally broken during DNA methylation, the DNA methyltransferase remains covalently bound to azacytosine and is unable to methylate the DNA (Stresemann and Lyko 2008). Furthermore, the entrapment of the DNA methyltransferases triggers DNA damage signalling which results in degradation of the methyltransferases (Stresemann and Lyko 2008). The action of DAC therefore leads to a global loss of methylation during DNA replication (Stresemann and Lyko 2008).

TSA is a HDAC inhibitor and acts by binding to the catalytic domains within class I and II HDACs (Drummond *et al.* 2005). Binding of TSA to the HDAC inhibits the enzyme's ability to deacetylate histones due to chelation of the zinc cation and displacement of the water molecule present in the active site of the enzyme (Drummond *et al.* 2005).

K562 and KG-1a cells lines were subcultured to $2x10^5$ cells/mL and were treated with either DAC (500 ng/mL; Sigma-Aldrich, USA) or TSA (200 ng/mL) or a combination of DAC and TSA. Cells were treated with DAC for 72 hours, during which time the cells would normally replicate 2-3 times, thus ensuring that the agent was incorporated into the DNA, leading to a loss of methylation after cell division. Additionally, both untreated and DAC treated cells were treated with TSA for 6 hours. Kasumi-1 cells were subcultured to $4x10^5$ cells/mL and were treated as described for K562 and KG-1a cell lines.

2.2 Cloning of Promoter/Distal Regions

Integrin promoter/distal regions were cloned into a pXPG plasmid, containing a luciferase gene, to use in reporter assays to determine the effects of RUNX1 on these regions.

2.2.1 Primer Design

Upstream regions of the integrin genes ITGB4, ITGB5, ITGA6, ITGAV and ITGB1 were cloned into the pXPG luciferase reporter plasmid (provided by Professor Peter Cockerill, Bert *et al*. 2000). Primers shown in *Table 2.1* were designed using the Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/) to amplify a region approximately 500-1000 bp upstream of the transcription start site of the integrin genes which incorporated the DNA sequence bound by RUNX1 in SKNO-1 cells in the ChIP-seq study by Martens *et al.* (2012) and putative RUNX1 binding sites identified using the MatInspector tool in the Genomatix bioinformatics suite (http://www.genomatix.de/). Additional primers also described in *Table 2.1* were designed either to amplify sub-regions of the already cloned integrin promoters or to mutate putative RUNX1 binding motifs. All primers were checked for specificity using the NCBI Blast program, nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The New England Biolabs® (NEB) cutter V2.0 program (http://nc2.neb.com/NEBcutter2/) was used to identify restriction enzymes that did not digest the DNA within the promoter regions but digested the pXPG plasmid once in the multiple cloning site. The recognition sites of the selected enzymes were added to the 5' end of the primer sequences along with five random nucleotides to increase digestion efficiency (Table 2.1).

2.2.2 PCR Amplification of Promoter/Distal Regions

2.2.2.1 PCR Amplification Using Phusion Taq

Genomic DNA (gDNA) was isolated from K562 cells using the AllPrep DNA/RNA Mini Kit (Qiagen, USA) and used as template DNA for the PCR amplification of ITGA6, ITGAV and ITGB1 promoter regions, and ITGB4 distal regions, while pXPG-ITGB5 plasmid was used as template DNA for the PCR amplification of ITGB5 Del-5 and Del-6, pXPG-ITGB4 plasmid was used as template DNA for the PCR amplification of ITGB4 Del-6 promoter region, and pXPG-ITGA6 was used as template DNA for the PCR amplification of ITGA6 Del-1 and ITGA6 Mutant promoter regions. A 20 µL reaction containing 1X Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England

Biolabs, USA) and 0.5 µM of primers (Table 2.1) was used to amplify DNA (20 ng gDNA, 1ng plasmid) following the manufacturer's instructions. DNA was amplified using a Veriti® 96 Well Thermal Cycler (Applied Biosystems™, USA) under the following cycling conditions: initial denaturation step of 30 seconds at 98˚C and then 35 cycles consisting of 98˚C for 10 seconds, 62-72˚C (depending on the primer set used) for 10 seconds and 72˚C for 20-30 seconds (30 seconds per kb of DNA to be amplified). Following the last cycle, samples were held at 72˚C for 7 minutes. PCR products were visualised by agarose gel electrophoresis and amplified promoter/distal regions were excised from the gels. PCR products were extracted and purified from the agarose gel using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Samples were eluted in 10-20 µL of Elution Buffer.

2.2.2.2 PCR Amplification Using GoTaq

pXPG-ITGB4 plasmid DNA (1 ng, Section 2.3.1) was used as template DNA for the PCR amplification of ITGB4 Del-2, Del-3, Del-4 and Del-5 promoter regions, while pXPG-ITGB5 plasmid DNA (1 ng, Section 2.3.1) was used as template DNA for the PCR amplification of ITGB5 Del-4 promoter region. A 20 µL reaction containing 1X GoTaq® Green Master Mix (Promega, USA) with 0.45 µM of primers (Table 2.1) was used to amplify DNA following the manufacturer's instructions. DNA was amplified using a Veriti® 96 Well Thermal Cycler (Applied Biosystems™, USA) under the following cycling conditions: initial denaturation step of 95˚C for 3 minutes and then 35 cycles consisting of 95˚C for 15 seconds, 52-62˚C (depending on primer set used) for 30 seconds and 72˚C for 30-60 seconds (1 minute per kb of DNA to be amplified). Following the last cycle, samples were held at 72˚C for 7 minutes. PCR products were visualised by agarose gel electrophoresis to verify a single product amplified. PCR products were purified using illustra GFX PCR DNA and Gel Band Purification Kit following the manufacturer's instructions and were eluted in 20 µL of Elution Buffer Type 4.

2.2.3 Ligation of Amplified DNA into pXPG

Purified PCR products and pXPG luciferase reporter plasmid were first digested overnight with the appropriate enzymes detailed in *Table 2.1*. Digested DNA was purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) following the manufacturer's instructions and samples were eluted in 20 µL of Elution Buffer Type 4. For ITGB4 Del-5 Fragment 1 (amplified using Forward1 and Reverse1

primers detailed in Table 2.1), PCR products were first digested with *SmaI* (New England Biolabs, USA) for 6 hours, followed by an overnight digestion with *XhoI* and *HindIII* enzymes (New England Biolabs, USA) to obtain different sized products which could be visualised by agarose gel electrophoresis. Digested ITGB4 Del-5 Fragment 1 PCR products were subjected to agarose gel electrophoresis using a 3% gel. Digested PCR products were electrophoresed at 100V for 1 hour and 40 minutes to allow the separation of digested PCR products at the sizes of 466 bp and 436 bp. Digested PCR products at the size of 466 bp were excised from the agarose gel, purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions and were eluted in 10 µL of Elution Buffer.

For the cloning of the ITGB4 distal regions, ITGB4 Del-6 was used as the vector and was digested with *BamHI* and *XhoI* enzymes (New England Biolabs, USA). The digested plasmid was purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) following the manufacturer's instructions and samples were eluted in 20 µL of Elution Buffer Type 4.

Purified digested PCR products and plasmid were quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA). A 10 µL reaction containing 1X T4 DNA Ligase Reaction Buffer (New England Biolabs, USA), 1 µL of T4 DNA Ligase (New England Biolabs, USA), 50 ng of plasmid (vector) and an appropriate volume of PCR products (insert) to ensure a 1:3 vector to insert molar ratio was used. The ligation reactions were incubated at room temperature overnight.

Promoter/ Distal Region	**Primer Sequences**	**Fragment Size**	**RE**
ITGB4 Del-2	Forward: 5' – TGCTACTCGAGCTGCTCTCAGAGGACTGACG – 3'	924 bp	*SmaI HindIII*
	Reverse: 5' – TGCTAAAGCTTCCCGTCCTGGACCTACCT – 3'		
ITGB4 Del-3	Forward: 5' – TGCTACTCGAGATGCAGCCGGTCTGACTC – 3'	223 bp	*XhoI HindIII*
	Reverse: 5' – TGCTAAAGCTTCCCGTCCTGGACCTACCT – 3'		
ITGB4 Del-4	Forward: 5' – TGCTACTCGAGATGCAGCCGGTCTGACTC – 3'	92 bp	*XhoI HindIII*
	Reverse: 5' – TGCTCAAGCTTAGGCGGGCAGCGCTTTAT – 3'		
ITGB4 Del-5	Forward1: 5' – TGCTACTCGAGCTGCTCTCAGAGGACTGACG – 3'	924 bp	*XhoI SmaI HindIII*
	Reverse1: 5' – TGCTAAAGCTTCCCGTCCTGGACCTACCT – 3'		
	Forward2: 5' – TGCTACCCGGGATGCAGCCGGTCTGACTC – 3'	223 bp	*SmaI HindIII*
	Reverse2: 5' – TGCTAAAGCTTCCCGTCCTGGACCTACCT – 3'		
ITGB4 Del-6	Forward: 5' – TGCTACTCGAGCTAGCCGATCGGGGCGCT – 3'	319 bp	*XhoI HindIII*
	Reverse: 5' – TGCTAAAGCTTCCCGTCCTGGACCTACCT – 3'		
ITGB4 Mutant	Forward: 5' – ATTAACCCGGGGCAGTCCGCGCA – 3'	170 bp	*SmaI BglII*
	Reverse: 5' – ATTAAAGATCTCCCGCGGCGCCCGCCCA – 3'		
	Forward: 5' – ATTAAAGATCTAGCCCTTTCCGGGGGGCGG – 3'	301 bp	*BglII HindIII*
	Reverse: 5' – TGCTAAAGCTTCCCGTCCTGGACCTACCT – 3'		
ITGB4 Distal-1	Forward: 5' – TGCTAGGATCCGAGGCGGCAGCTCATTGT – 3'	334 bp	*BamHI XhoI*
	Reverse: 5' – TGCTACTCGAGGTGCCATTTCAGACCACCT – 3'		
ITGB4 Distal-2	Forward: 5' – TGCTAGGATCCCTAGGGCTCGATTTCCAAAG – 3'	332 bp	*BamHI XhoI*
	Reverse: 5' – TGCTACTCGAGCCTCCTGAGTAGCTGGGAAT – 3'		
ITGB5 Del-4	Forward: 5' – TGCTACTCGAGCGTCTCGGAGCCCAAGTC – 3'	125 bp	*XhoI HindIII*
	Reverse: 5' – TGCTAAAGCTTCCGGGACTCCTAGTGT – 3'		
ITGB5 Del-5	Forward: 5' – TGCTACTCGAGCTGCTCTCCCTCTCGCAGT – 3'	464 bp	*XhoI HindIII*
	Reverse: 5' – TGCTAAAGCTTGACTTGGGCTCCGAGACG – 3'		
ITGB5 Del-6	Forward: 5' – TGCTACTCGAGTACCCGGAGCAGCCCGCT – 3'	77 bp	*XhoI HindIII*
	Reverse: 5' – TGCTAAAGCTTCCGGGACTCCTAGTGT – 3'		
ITGA6	Forward: 5' – TGCTACTCGAGCATCCTTGACTTGCGTGACT – 3'	939 bp	*XhoI HindIII*
	Reverse: 5' – TGCTAAAGCTTCGACAGGTAGAGCAAGCACA – 3'		

ITGA6 Del-1	Forward: 5' – TGCTACTCGAGCAGCTGGAGACGCCAGAG – 3' Reverse: 5' – TGCTAAAGCTTCGACAGGTAGAGCAAGCACA – 3'	404 bp	*XhoI* *HindIII*
ITGA6 Mutant	Forward: 5' – TGCTACTGGAGCATCCTTGACTTGCGTGACT – 3' Reverse: 5' – TGCTAGATATCTGCCGAGTAGCACAGAGCGA – 3'	460 bp	*XhoI* *EcoRV*
	Forward: 5' – TGCTAGATATCATTCTGTCCACAGAGGGCGG – 3' Reverse: 5' – TGCTAAAGCTTCGACAGGTAGAGCAAGCACA – 3'	495 bp	*EcoRV* *HindIII*
ITGAV	Forward: 5' – TGCTACTCGAGACAACAGTCGCACGGAAGTT – 3' Reverse: 5' – TGCTAAAGCTTCAAGAGGGCTGAGCTTCG – 3'	545 bp	*XhoI* *HindIII*
ITGB1	Forward: 5' – TGCTACTCGAGTTAGCCAGTTCCCTTCCAGA – 3' Reverse: 5' – TGCTAAAGCTTCGGCGGCTTTAAGTGCTG – 3'	809 bp	*XhoI* *HindIII*

Table 2.1 – Primers used for molecular cloning. This table details the primers used to clone the various regions of the integrin genes. The restriction enzyme recognition site within the primers are underlined. Expected PCR product size as well as the restriction enzymes used to digest the PCR products are shown.

2.2.4 Plasmid Transformation and Isolation

Ligated DNA (5 µL) was transformed into One Shot® TOP10 Chemically Competent *E.coli* Cells (25 µL; Life Technologies, USA) or JM109 Competent cells (25 µL; Promega, USA) following the manufacturer's instructions. Transformed cells were plated on agar plates containing 100 µg/mL ampicillin (Sigma-Aldrich, USA) and incubated overnight at 37˚C. Plasmid DNA from 6-12 selected colonies was isolated and purified using either Wizard® *Plus* SV Minipreps Purification System (Promega, USA) or QIAprep Spin Miniprep Kit (Qiagen, USA) following the manufacturer's instructions. DNA was eluted in 30-50 µL of nuclease-free water or Buffer EB and 500-1000 ng of plasmid DNA was analysed for successfully ligated recombinant plasmid by restriction enzyme digestion and agarose gel electrophoresis.

2.2.5 DNA Sequencing

Plasmid DNA identified as containing insert by restriction enzyme digestion (Section 2.2.4) was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) to verify the correct insert sequence was cloned and to ensure there were no mutations in putative RUNX1 binding motifs. A 10 µL reaction consisting of 1.75 µL of BigDye® Terminator sequencing buffer, 0.25 µL of BigDye® Terminator, 0.528 µM of pXPG reverse primer (5' TGGAAGACGCCAAAAACATAAAG 3') or 0.528 µM of forward primer used to clone the specific integrin region (Table 2.1) and 100-200 ng of purified plasmid DNA was used for the dye-terminator sequencing. Dye-terminator sequencing was performed using a Veriti Thermal Cycler (Applied Biosystems, USA) under the following conditions: initial denaturation step of 96˚C for 1 minute and then 25 cycles consisting of 96˚C for 10 seconds, 50˚C for 5 seconds and 60˚C for 4 minutes. Products were purified using the Agencourt® CleanSEQ® kit (Agencourt Bioscience Corporation, USA) and were eluted in 40 µL of water. Purified DNA was sequenced using an ABI Prism® 310 Genetic Analyzer (Applied Biosystems, USA) and sequencing data was recovered using 310 Data Collection and Sequence Analysis Software (Applied Biosystems, USA). Sequencing files were analysed using the computer software program Sequencher 4.10.1 (Gene Codes Corporation, USA).

2.3 Luciferase Reporter Assays

Luciferase reporter assays were used to monitor RUNX1 effects on integrin promoter/enhancer activity in K562 cells.

2.3.1 Plasmid Preparation

Plasmids summarised in *Table 2.3* were transformed into One Shot® TOP10 Chemically Competent *E.coli* Cells (25 µL; Life Technologies, USA; section 2.2.4) following the manufacturer's instructions. Starter cultures were first made by inoculating 1 mL of L broth (containing the appropriate antibiotic) with a single isolated colony or from a glycerol culture. Starter cultures were incubated for 6 hours at 37˚C with shaking, before inoculating 99 mL of L broth (containing appropriate antibiotic). Cultures were incubated overnight at 37˚C with shaking. The plasmid DNA was extracted and purified using either the Qiagen Plasmid Maxi Kit (Qiagen, USA) or the Qiagen Plasmid *Plus* Maxi Kit (Qiagen, USA) following the manufacturer's instructions. Plasmid DNA was eluted in 50-100 µL of either Tris/EDTA (TE) Buffer or Buffer EB and was quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA). Plasmid DNA was diluted to 1000 ng/µL and re-quantified. Purified plasmid DNA was stored at -20˚C.

Plasmid Name	Promoter/Distal Region Name	Description	Source
pXPG	-	- Size = 6087 bp - Ampicillin resistance gene	Bert *et al.* (2000)
pGL3-Control	-	- Size = 5256 bp - Ampicillin resistance gene - Contains *SV40* promoter and enhancer sequences which results in strong expression of luciferase gene	Promega
RcCMV	-	- Size = 5446 bp - Ampicillin resistance gene	Invitrogen (Life Technologies)
pCMV5-AML1B (RUNX1)	-	- Size = ~5.8 kb - Ampicillin resistance gene	Addgene Meyers *et al.* (1995)
pCMV5-AML1-ETO (RUNX1-ETO)	-	- Size = ~9 kb - Ampicillin resistance gene	Addgene Meyers *et al.* (1995)
pXPG-ITGB4	ITGB4	- Size = 7406 bp - Contains -1199 bp to +144 bp of human ITGB4 gene - Ampicillin resistance gene	Cloned by Jessica Phillips (2012)
pXPG-ITGB4 Del-2	ITGB4 Del-1	- Size = 6965 bp - Contains -758 bp to +144 bp of ITGB4 gene - Ampicillin resistance gene	Cloned by Jessica Phillips (2012)
pXPG-ITGB4 SmaI	ITGB4 Del-2	- Size = 6514 bp - Contains -295 bp to +144 bp of human ITGB4 gene -Ampicillin resistance gene	Refer to Section 2.2
pXPG-ITGB4 Del-3	ITGB4 Del-3	- Size = 6264 bp - Contains -57 bp to +144 bp of human ITGB4 gene - Ampicillin resistance gene	Refer to Section 2.2

pXPG-ITGB4 Del-4	ITGB4 Del-4	- Size = 6133 bp - Contains -57 bp to +13 bp of human ITGB4 gene - Ampicillin resistance gene	Refer to Section 2.2
pXPG-ITGB4 Del-6	ITGB4 Del-5	- Size = 6730 bp - Contains -758 bp to -290 bp and -57 bp to +144 bp of human ITGB4 gene - Ampicillin resistance gene	Refer to Section 2.2
pXPG-ITGB4 Del-8	ITGB4 Del-6	- Size = 6382 bp - Contains -175 bp to +144 bp of human ITGB4 gene - Ampicillin resistance gene	Refer to Section 2.2
pXPG-ITGB4 Mutant2	ITGB4 Mutant	- Size = 6514 bp - Contains -295 bp to +144 bp of human ITGB4 gene - Possesses BglII recognition sequence mutation at site -141 bp to -136 bp of human ITGB4 gene Ampicillin resistance gene	Refer to Section 2.2
pXPG-ITGB4 Del-8 Enhancer 1	ITGB4 Distal-1	- Size = 6730 bp - Contains -13,815 bp to -14,178 bp and -175 bp to +144 bp of human ITGB4 gene -Ampicillin resistance gene	Refer to Section 2.2
pXPG ITGB4 Del-8 Enhancer 2	ITGB4 Distal-2	-Size = 6698 bp - Contains -12,077 bp to -12,408 bp and -175 bp to +14 bp of human ITGB4 gene -Ampicillin resistance gene	Refer to Section 2.2
pXPG-ITGB5	ITGB5	- Size = 6633 bp - Contains -496 bp to +74 bp of human ITGB5 gene - Ampicillin resistance gene	Originally cloned into pXP1 by Paulynn Chin (2009) and sub-cloned into pXPG by Jessica Phillips (2014)

pXPG-ITGB5 Del-1	ITGB5 Del-1	- Size = 6590 bp - Contains -453 bp to +74 bp of human ITGB5 gene - Ampicillin resistance gene	Cloned by Jessica Phillips (2012)
pXPG-ITGB5 Del-2	ITGB5 Del-2	- Size = 6546 bp - Contains -409 bp to +74 bp of human ITGB5 gene - Ampicillin resistance gene	Cloned by Jessica Phillips (2012)
pXPG-ITGB5 Del-3	ITGB5 Del-3	- Size = 6445 bp - Contains -308 bp to +74 bp of human ITGB5 gene - Ampicillin resistance gene	Cloned by Jessica Phillips (2012)
pXPG-ITGB5 Del-5	ITGB5 Del-4	- Size = 6166 bp - Contains -20 bp to +74 bp of human ITGB5 gene - Ampicillin resistance gene	Refer to Section 2.2
pXPG-ITGB5 Del-6	ITGB5 Del-5	- Size = 6505 bp - Contains -453 bp to -12 bp of human ITGB5 gene - Ampicillin resistance gene	Refer to Section 2.2
pXPG-ITGB5 Del-7	ITGB5 Del-6	- Size = 6118 bp -Contains +20 bp to +74 bp of human ITGB5 gene - Ampicillin resistance gene	Refer to Section 2.2
pXPG-ITGA6	ITGA6	- Size = 6980 bp - Contains -675 bp to +242 bp of human ITGA6 gene - Ampicillin resistance gene	Refer to Section 2.2
pXPG-ITGA6 Del-1	ITGA6 Del-1	- Size = 6445 bp - Contains -140 bp to +242 bp of human ITGA6 gene - Ampicillin resistance gene	Refer to Section 2.2
pXPG-ITGA6 Mutant	ITGA6 Mutant	- Size = 6980 bp	Refer to Section 2.2

		- Contains -675 bp to +242 bp of human ITGA6 gene - Possesses *EcoRV* recognition sequence mutation at site -237 bp to -232 bp of human ITGA6 gene - Ampicillin resistance gene	
pXPG-ITGAV	ITGAV	- Size = 6586 bp - Contains -398 bp to +125 bp of human ITGAV gene - Ampicillin resistance gene	Refer to Section 2.2
pXPG-ITGB1	ITGB1	- Size = 6850 bp - Contains -485 bp to +302 bp of human ITGB1 gene - Ampicillin resistance gene	Refer to Section 2.2

Table 2.2 – Details and source of plasmids used in reporter assays.

2.3.2 Transfection of Myeloid Cell Lines

K562 cells (4.5 x 10^6 cells in 300 µL of RPMI containing 20% FCS) were transfected with purified plasmid DNA (Section 2.3.1) using electroporation. Briefly, plasmid DNA (1-10 µg) and cells were added to a Gene Pulser® electroporation cuvette (4 mm; Bio-Rad, USA) and electroporated using a Gene Pulser® Xcell™ system (Bio-Rad, USA) at 270 V with a capacitance of 950 µFarad and a resistance of infinity. Next, 1 mL of RPMI (10% FCS) was added to the cuvette and cells were allowed to recover for 5-10 minutes at room temperature. Cells were then added to a tissue culture flask containing 3.7 mL RPMI medium (10% FCS) and were incubated in a humidified incubator at 37˚C with 5% CO_2 for 24 hours. Transfections were performed in duplicate and combined after electroporation. Supplementation with RcCMV plasmid was used to ensure equal amounts of plasmid DNA were transfected into the cells.

To isolate the protein from transfected cells, cells were first centrifuged at 500 x *g* for 5 minutes and the cell pellet was washed with 10 mL of phosphate buffered saline (PBS; Thermo Fisher Scientific, USA). Cells were centrifuged again and the cell pellet was resuspended in 100 µL of 1X Lysis Buffer (Promega, USA). Cell lysate was either stored at -20˚C for later analysis or incubated on ice for approximately 2 minutes. Cell lysate was then centrifuged at 13,000 x *g* for 5 minutes, the supernatant was retained and stored at -20˚C for later analysis.

2.3.3 Determination of Protein Concentration by Bradford Assay

Bradford assay was used to determine protein concentration of cell lysates described in *Section 2.3.2*. Briefly, an aliquot of cell lysate was first diluted 1:10 to be measured for protein concentration. A standard curve was generated using bovine serum albumin (BSA; New England Biolabs, USA) at concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL. The Protein Assay Dye Reagent Concentrate (Bio-Rad, USA) was diluted 1:5 and 990 µL was added to 10 µL of diluted cell lysate and BSA standards. Samples were mixed well and allowed to stand at room temperature for 5 minutes. Protein absorbance was measured at 595 nm using a SpectraMax® Plus^{384} Microplate Reader Spectrophotometer (Molecular Devices, USA) and BSA standard curves were constructed using Microsoft® Excel to determine protein concentration of cell lysate samples.

2.3.4 Analysis of Promoter and Enhancer Activity by Luciferase Assay

The effects of RUNX1 and RUNX1-ETO transcription factors on integrin promoter or enhancer activity was analysed using the Luciferase Reporter System (Promega, USA). Firstly, 50 µg of protein extracts (10 µg was used for ITGAV and ITGB1 samples due to high luminescence) were added to each well of a 96-well plate (Greiner Bio-one, Germany) and assays were performed in duplicate. Samples were made up to a total volume of 100 µL with 1X Lysis buffer (Promega, USA) and 100 µL of Luciferase reagent was added to each well. The light emitted from the luciferase-cataylsed chemiluminescent reaction, in relative light units, was measured using a 2.5 second integration time on a Veritas™ Microplate Luminometer (Turner Biosystems, USA), and therefore represent relative luciferase activity/units.

2.4 Analysis of mRNA Levels by Reverse Transcription Quantitative PCR

Integrin and *RUNX1* expression was analysed in leukaemic cell lines by the detection of mRNA levels using real-time reverse transcription quantitative PCR (RT-qPCR).

2.4.1 RNA Extraction

RNA was extracted from cells using TRI Reagent® (Sigma-Aldrich, USA) following the manufacturer's instructions. Briefly, cell pellets (approximately 4 x 10^6 cells) were lysed with 500 µL of TRI Reagent® and incubated at room temperature for 5 minutes. Samples underwent phase separation by the addition of 100 µL of chloroform. Samples were shaken vigorously for 15 seconds and incubated at room temperature for 10 minutes. After centrifugation at 13,000 x *g* for 15 minutes at 4˚C, the top aqueous phase containing RNA was transferred to a new tube and RNA was precipitated overnight at -20˚C by the addition of 250 µL of isopropanol. Precipitated RNA collected by centrifugation was washed with 75% ethanol and resuspended in diethylpyrocarbonate-treated water. RNA was quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA).

2.4.2 cDNA Synthesis

RNA samples (up to 1 µg) were first incubated with 1 unit of DNase I (Sigma-Aldrich, USA) in 1X First Strand Buffer (Life Technologies, USA) for 30 minutes at 37˚C. DNase

I enzyme was heat-inactivated at 75˚C for 5 minutes, then 5 µM of oligo dT (Geneworks, AUS) was added to the samples and incubated at 70˚C for 10 minutes. RNA was reverse transcribed using 100 units of SuperScript® III Reverse Transcriptase enzyme (Life Technologies, USA) in 1X First Strand Buffer containing 10mM DTT (Life Technologies, USA) and 1mM of dNTPs (Sigma-Aldrich, USA) at 42˚C for 50 minutes. SuperScript® III enzyme was heat-inactivated at 70˚C for 15 minutes and cDNA samples were stored at -20˚C.

Alternatively, in some experiments cDNA was synthesised using the iScript™ cDNA synthesis kit (Bio-Rad, USA) following the manufacturer's instructions.

2.4.3 RT-qPCR

RT-qPCR was performed as described previously (Qadi *et al.* 2016). cDNA synthesised in *Section 2.4.2* was used as template DNA to detect the expression of genes described in *Table 2.3*. Primers shown in *Table 2.3* were designed to amplify a region 50-150 bp of mRNA of selected integrin genes using the Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/). All primers were checked for specificity using the NCBI Blast program, nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A 25 µL reaction containing 1X QuantiTect SYBR® Green PCR Master Mix (Qiagen, USA) and 0.38 µM of each forward and reverse primer (Table 2.3), was used to amplify cDNA (50 ng) following the manufacturer's instructions. cDNA was amplified using a Corbett Rotor-Gene Cycler (Qiagen, USA) under the following conditions: initial denaturation step at 95˚C for 15 minutes and then 40 cycles consisting of 94˚C for 15 seconds and 60˚C for 60 seconds. The amplification of human GAPDH mRNA or β2-Microglobulin (primers detailed in Table 2.3) was also conducted in parallel to all PCRs to normalise for discrepancies in cDNA synthesis and RNA input. To ensure a single PCR product was generated, PCR product melt curves were analysed for a single peak and the products were visualised by agarose gel electrophoresis. To determine copy number from the cycle threshold values from the amplification plots, a standard curve was generated for each primer pair with serial dilutions of the PCR product.

Gene	Primer Sequence	Product Size
GAPDH	Forward: 5’ – AAATATGATGACATCAAGAAGG – 3’	67 bp
	Reverse: 5’ – AGCCCAGGATGCCCTTGAGGG – 3’	
β2-Microglobulin	Forward: 5’ – ACTGAATTCACCCCCACTGA – 3’	114 bp
	Reverse: 5’ – CCTCCATGATGCTGCTTACA – 3’	
RUNX1	Forward: 5’ – CACCTACCACAGAGCCATCA – 3’	109 bp
	Reverse: 5’ – CTCGGAAAAGGACAAGCTCC – 3’	
RUNX1-ETO	Forward: 5’ – AATCACAGTGGATGGGCCC – 3’	87 bp
	Reverse: 5’ – TGCGTCTTCACATCCACAGG – 3’	
ITGB4	Forward: 5’ – TTAAGAGAGCCGAGGAGGTG – 3’	138 bp
	Reverse: 5’ – GGCAGTCCTTCTTCTTGTGC – 3’	
ITGB5	Forward: 5’ – AGCCAGAGTGTGGAAACACC – 3’	105 bp
	Reverse: 5’ – CAAGCAGCTTCCAGATAGCC – 3’	
ITGA6	Forward: 5’ – CCAAAAATTACTTTGGGGCTAA – 3’	122 bp
	Reverse: 5’ – TCAGCTTTCATATCTATTCAGTCTCTG – 3’	
ITGAV	Forward: 5’ – TGCAAAATGTAATGATGAGCTTG – 3’	102 bp
	Reverse: 5’ – CCAAGAATGCAAACAAGGTG – 3’	
ITGB1	Forward: 5’ – TTTTGAAAATAATGTTGTAATTCATGC – 3’	104 bp
	Reverse: 5’ – GAAAAGGTCAAAAAGGCACAA – 3’	

Table 2.3 – RT-qPCR primers. Detailed are the primers used in RT-qPCR to detect the mRNA levels of specific genes and the expected size of the resultant PCR product.

2.5 Analysis of Protein by Western Blot Analysis

Protein expression in leukaemic cell lines was analysed using Western Blot analysis.

2.5.1 Cytosolic and Nuclear Protein Extraction

Cytosolic and nuclear extracts were prepared from cells using a modified version of the method described by (Schreiber *et al.* 1989). Briefly, cells (up to 1.25 x 10^7 cells) were pelleted at 500 x *g* for 5 minutes at 4˚C and washed in 10 mL of ice-cold PBS (Thermo Fisher Scientific, USA). Cell pellets were resuspended in 1 mL of ice-cold Lysis Buffer (10 mM Tris at pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA at pH 8.0 and 0.5% Igepal) and incubated on ice for 5 minutes. Nuclei were pelleted by centrifugation at 500 x *g* for 5 minutes at 4˚C and supernatant containing cytoplasmic proteins was transferred to a new tube and retained. The nuclei were washed in 1 mL of ice-cold Igepal-free buffer (10 mM Tris at pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 0.1 mM EDTA at pH 8.0) and centrifuged at 500 x *g* for 5 minutes at 4˚C. Nuclei were resuspended in 25 µL of Nuclei Lysis Buffer (400 mM NaCl, 7.5mM $MgCl_2$, 0.2mM EDTA at pH 8.0 and 1mM DTT) supplemented with 1 µL aprotinin (2 mg/mL; Sigma-Aldrich, USA) leupeptin (1 mg/mL; Amersham Biosciences, USA) and Complete EDTA-Free Protease Inhibitor Cocktail (Roche Applied Science, Switzerland) and incubated on ice with shaking for 15 minutes. The nuclear lysate was centrifuged at 13,000 x *g* for 5 minutes at 4˚C and the supernatant containing nuclear proteins was transferred to a new tube and retained. Protein extracts were quantified using the Bradford assay (Section 2.3.4).

2.5.2 SDS-PAGE and Western Blotting

Protein extracts prepared in *Section 2.5.1* (10-20 µg) were first denatured at 95˚C for 5 minutes in sample loading buffer (62.5 mM Tris HCl pH 6.8, 10% Glycerol, 2% SDS, 0.005% Bromophenol blue, 10% β-mercaptoethanol). Proteins were then separated by SDS-PAGE through a 4-20% Mini-PROTEAN® TGX™ pre-cast gel (Bio-Rad, USA) in SDS-PAGE Running Buffer (192 mM Glycine, 25 mM Tris and 0.1% SDS) and were transferred to 0.45 µM nitrocellulose membrane (Bio-Rad, USA) through a wet transfer in Western Transfer Buffer (192 mM Glycine, 25 mM Tris and 20% methanol). Proteins were transferred overnight at 20 V at 4˚C and were then subjected to western blot analysis using the antibodies described in *Table 2.4* with the corresponding peroxidase-conjugated secondary antibodies. Proteins were visualised using the Supersignal West Pico

Chemiluminescent kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. For the analysis of additional proteins, membranes were stripped using Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific, USA) following the manufacturer's instructions and were re-probed.

Antibody	Type	Dilution	Supplier
Anti-RUNX1 (H-65)	Rabbit polyclonal	1:1000	Santa Cruz Biotechnology (sc-28679)
Anti-RUNX1 (N-20)	Goat polyclonal	1:500	Santa Cruz Biotechnology (sc-8563)
Anti-ETO (C-20)	Goat polyclonal	1:500	Santa Cruz Biotechnology (sc-9737)
Anti-Histone H3	Rabbit polyclonal	1:700	Abcam (ab1791)
2º Anti-Goat HRP	Rabbit polyclonal	1:2000	Dako (P0449)
2º Anti-Rabbit HRP	Goat polyclonal	1:1000	Dako (P0448)

Table 2.4 – Antibodies used for Western Blot analysis.

2.6 DNA Methylation Analysis

DNA methylation of integrin promoter regions was determined using the bisulphite sequencing technique as developed by Frommer *et al*. (1992). Bisulphite sequencing involves the treatment of DNA with bisulphite which converts cytosine into uracil while methylated cytosines are resistant to treatment and therefore remain as cytosines (Frommer *et al.* 1992). The bisulphite treated DNA is then amplified by PCR using primers to a specific region of DNA to be analysed, and then PCR products can be cloned and sequenced to provide methylation maps of single DNA molecules (Frommer *et al.* 1992). During the PCR process, all uracil and thymines are amplified as thymine, while methylated cytosines are amplified as cytosines (Frommer *et al.* 1992). Methylated and unmethylated CpGs are determined by comparing bisulphite-converted sequences to a reference sequence.

2.6.1 DNA Isolation and Bisulphite Conversion

Genomic DNA was isolated and purified from K562, KG-1a and Kasumi-1 cells using the Allprep DNA/RNA Mini kit (Qiagen, USA) or QIAamp DNA Blood Mini Kit (Qiagen, USA) following the manufacturer's instructions. Genomic DNA from K562, KG-1a and Kasumi-1 cell lines was bisulphite converted using the EZ DNA Methylation Gold kit (Zymo Research, USA) following the manufacturer's instructions and bisulphite converted DNA was eluted in 10 µL of M-Elution Buffer. Human DNA samples originating from the Royal Hobart Hospital were also analysed. Genomic DNA from a 68 year old female individual with t(8;21)-positive leukaemia (LK7770) and from non-leukaemic age and sex match controls were bisulphite converted following the protocol used for the leukaemic cell lines. Use of these samples were approved by Human Research Ethics Committee (Tasmanian network), reference number: H8551.

2.6.2 Primer Design

Primers used for the PCR amplification of bisulphite converted DNA were designed using the MethPrimer program (http://www.urogene.org/methprimer/index.html) which can locate CpG islands within a query sequence and design primers around the predicted CpG islands (Li and Dahiya 2002).

2.6.3 PCR of Bisulphite Converted DNA

Bisulphite converted gDNA (20 ng) was amplified by PCR using the EPIK Amplification kit (Bioline, USA). A 20 µL reaction containing 1X EPIK amplification mix and 0.4 µM of primers (Table 2.5) was used to amplify DNA following the manufacturer's instructions. DNA was amplified using a Veriti® 96 Well Thermal Cycler (Applied Biosystems™, USA) under the following cycling conditions: initial denaturation step of 2 minutes at 95ºC and then 45 cycles consisting of 15 seconds at 95ºC, 15 seconds at 54ºC and 30-60 seconds at 72ºC (30 seconds per 500 bp to be amplified).

2.6.4 Molecular Cloning

PCR products generated from bisulphite converted DNA as described in *Section 2.6.3* were visualised by gel electrophoresis and were excised from the gels. PCR products were extracted and purified from the agarose gel using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Samples were eluted in 10-20 µL of Elution Buffer. Purified PCR products were ligated into a pGEM®-T Easy Vector (Promega, USA) following the protocol described in *Section 2.2.3* and ligated DNA was transformed into One Shot® TOP10 Chemically Competent *E.coli* Cells (25 µL; Invitrogen, USA) following the manufacturer's instructions. Transformed cells were plated on agar plates, containing 100 µg/mL ampicillin (Sigma-Aldrich, USA) and 50 µg/mL X-gal (Promega, USA), to allow blue/white colour selection of recombinant plasmids, and incubated overnight at 37˚C.

Plasmid DNA from up to 10 white colonies was isolated and purified using the QIAprep Spin Miniprep Kit (Qiagen, USA) following the manufacturer's instructions. DNA was eluted in 30 µL Buffer EB and 100-1000 ng of plasmid DNA was analysed for successfully ligated recombinant plasmid by restriction enzyme digestion and agarose gel electrophoresis. Plasmid DNA identified as containing the insert by restriction enzyme digestion was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following the protocol described in *Section 2.2.5* using the sp6 reverse primer (5' – TATTTAGGTGACACTATAGAAT – 3').

Sequencing was analysed using BiQ Analyzer software (Max Planck Institute Informatik, Germany) and bubble maps were generated using CpG Bubble Chart Generator, Version 20061209 Alpha (created by Mark A. Miranda).

Bisulphite PCR Primers	Primer Sequence	Product Size	Gene Position
ITGB4 Fragment A	Forward: 5' – GTGAATTGTAGAGTAGAGTTGGGA – 3'	792 bp	-516 bp to +276 bp
	Reverse: 5' – AAACCTATCAACCTTCAAAAAAAA – 3'		
ITGB4 Fragment B	Forward: 5' – GGTAGTAGTAGTAGTATAGTTTGGTG – 3'	666 bp	-390 bp to +276 bp
	Reverse: 5' – AAACCTATCAACCTTCAAAAAAAA – 3'		
ITGA6 Fragment A	Forward: 5' – AATAGGTTGTTTAGGTTATGAGGTT – 3'	569 bp	-329 bp to +240 bp
	Reverse: 5' – ACAAATAAAACAAACACAACTACCC – 3'		
ITGA6 Fragment B	Forward: 5' – GGGTAGTTGTGTTTGTTTTATTTGT – 3'	605 bp	+216 bp to +821 bp
	Reverse: 5' – AAAACCTAAACCATCCTAAAACTAC – 3'		

Table 2.5 – Primers used for PCR amplification of bisulphite converted DNA. Detailed are the primers used for the PCR amplification of ITGB4 and ITGA6 CpG rich regions. Primers were designed using the MethPrimer program (Li and Dahiya 2002). Locations of the regions are shown as well as the expected PCR products size.

2.7 Chromatin Analysis

Chromatin immunoprecipitation (ChIP) analysis was used to determine if RUNX1 binds to the endogenous integrin promoters/distal regions in leukaemic cell lines, and to determine histone H3 occupancy and acetylation at integrin promoter regions.

2.7.1 ChIP Assay

ChIP assays were performed as previously described (Brettingham-Moore *et al.* 2008). To cross-link proteins to DNA, KG-1a and Kasumi-1 cells (15 mL at 5.0 x 10^5 cells/mL) were treated with 1% formaldehyde for 8 minutes at room temperature on a rotary wheel. To halt the cross-linking process, 0.125 M glycine was added and cells were incubated at room temperature for 10 minutes on a rotary wheel. Cells were recovered by centrifuging at 500 x *g* for 5 minutes at 4˚C and washed twice in 10 mL of ice-cold PBS at 500 x *g* for 5 minutes at 4˚C. Nuclei were extracted following the protocol described in *Section 2.5.1* and nuclei (2.0 x 10^6) were resuspended in 200 µL of SDS Lysis Buffer (1% SDS, 10 mM EDTA and 50 mM Tris pH 8.1; Merck Millipore, USA) supplemented with 0.4% of Roche protease inhibitor cocktail (Roche Applied Science, Switzerland). Nuclei were incubated on ice for 10 minutes and were sonicated using a Bioruptor® Plus sonication device (Diagenode, Belgium) on high for 5 rounds of 10 cycles of 30 seconds ON and 30 seconds OFF, which was empirically determined to fragment the DNA into sizes between 100-500 bp. Sonicated nuclei were centrifuged twice at 13,000 x *g* for 5 minutes at 4˚C to remove debris. ChIP Dilution Buffer (1 mL, 0.01% SDS, 1.1% Triton, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1 and 167 mM NaCl; Merck Millipore, USA) supplemented with 0.1% Roche protease inhibitor cocktail was added and samples were pre-cleared with 60 µL of salmon sperm DNA/protein A agarose slurry (Merck Millipore, USA) for 2 hours at 4˚C on a rotary wheel. Antibodies described in *Table 2.6* were added to 450 µL of the pre-cleared samples, which were made up to 1 mL with the addition of ChIP Dilution Buffer supplemented with 0.1% Roche protease inhibitor cocktail, and incubated overnight at 4˚C on a rotary wheel, while 100 µL of the pre-cleared sample was retained as the Total Input (TI) fraction.

Immune complexes were recovered by incubation with 60 µL of salmon sperm DNA/protein A agarose slurry for 2 hours at 4˚C on a rotary wheel and samples were centrifuged at 500 x *g* for 1 minute at 4ºC to pellet the immune complex bound protein A

agarose beads. The supernatant was discarded and the beads were washed sequentially with 1 mL of Low Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1 and 150 mM NaCl; Merck Millipore, USA), High Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1 and 500 mM NaCl; Merck Millipore, USA), LiCl wash buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA and 10 mM Tris pH 8.1; Merck Millipore, USA), and twice with TE Buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0; Merck Millipore, USA). The immune complexes were eluted in 410 µL of Elution Buffer (1% SDS and 10 mM $NaHCO_3$). To reverse the cross-links, samples (380 µL) were incubated overnight at 65ºC with the addition of Proteinase K (20 µg; Qiagen, USA) and 4M NaCl (20 µL or 10 µL for TI sample).

DNA was purified using phenol/chloroform extraction (50% phenol, 50% chloroform) and precipitated overnight at -20ºC with 10% volume of 3M sodium acetate and 2.5X volume of 100% ethanol. DNA was washed 3 times in 70% ethanol, resuspended in 50 µL of water and 5 µL of DNA was used in qPCR following the protocol described in *Section 2.4.3*, using primers described in *Table 2.7*. Primers were designed to amplify a region of 50-190 bp of the ITGB4 and ITGA6 promoter/distal regions using the Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/). All primers were checked for specificity using the NCBI Blast program, nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Antibody	Type	Amount Used	Supplier
Anti-Histone H3	Rabbit polyclonal	2 µg	Abcam (ab1791)
Anti-Acetyl Histone H3	Rabbit polyclonal	4 µg	Merck Millipore (06-599)
Anti-RUNX1 (N-20)	Goat polyclonal	5 µg	Santa Cruz Biotechnology (sc-8563)

Table 2.6 – Details and source of antibodies used for ChIP assays.

Promoter/Distal Region	Primer Sequences	Fragment Size
Rhodopsin	Forward: 5’ – CCAATCTCCCAGATGCTGAT – 3’	54 bp
	Reverse: 5’ – TAAAGTGACCTCCCCCTCCT – 3’	
ITGB4 Promoter	Forward: 5’ – CTCGGACAGTCCCTGCTC – 3’	53 bp
	Reverse: 5’ – GCTGCCGCTAGGAGATGG – 3’	
ITGA6 Promoter	Forward: 5’ – GCGTCCTCGTCACTTGATAA – 3’	56 bp
	Reverse: 5’ – AATGAGCCCGTTGTTCTCTG – 3’	
ITGB4 Distal-1	Forward: 5’ – TGAAACGGGTTTCCCAGAC – 3’	64 bp
	Reverse: 5’ – ATCGCCAAAGATCATGAAGG – 3’	
ITGB4 Distal-2	Forward: 5’ – GCTATTGAGCCTGGTGCAGT – 3’	189 bp
	Reverse: 5’ – CCTCCTGAGTAGCTGGGAAT – 3’	

Table 2.7 – Primers used for ChIP analysis. Detailed are primer sequences used for qPCR for ChIP analysis and the expected sized PCR product.

Chapter 3

Identifying Integrin Genes Regulated by RUNX1

3.1 Introduction

3.1.1 Importance of RUNX1 in Haematopoiesis

The RUNX1 transcription factor plays a vital role in haematopoiesis and in addition is one of the most commonly disrupted genes in leukaemia. Point mutations, gene amplification and chromosomal translocations of *RUNX1* have all been reported in individuals with leukaemia (Dal Cin *et al.* 2001, Harewood *et al.* 2003, Miyoshi *et al.* 1995, Niini *et al.* 2000, Osato *et al.* 1999, Preudhomme *et al.* 2009, Song *et al.* 1999, Streubel *et al.* 2001). Disruption to *RUNX1* contributes to the development of leukaemia due to altered regulation of its target genes. Therefore, the identification of genes and biological pathways targeted by RUNX1, and the mechanism by which it regulates gene expression, is crucial for understanding its role in haematopoiesis, as well as its role in leukaemia development, and in identifying potential biomarkers or therapeutic targets for the disease. Yet, while many RUNX1 target genes have been identified through candidate gene analyses, the full repertoire of RUNX1 controlled genes remains to be determined.

3.1.2 RUNX1 Regulation of Integrin Genes

Through genome-wide studies, potential RUNX1 target genes have been identified and many of these have been found to be involved in cell signalling and cell adhesion. Further, a number of studies have demonstrated regulation of integrin genes by RUNX transcription factors. The ITGAL integrin gene is regulated by RUNX1 and RUNX3 in lymphoid and myeloid cells, respectively (Puig-Kröger *et al.* 2000, Puig-Kröger *et al.* 2003). Additionally, RUNX3 regulates the ITGA4 integrin gene in mature monocyte-derived dendritic cells (Domínguez-Soto *et al.* 2005). Interestingly, expression of the RUNX1-ETO fusion protein, produced from the t(8;21) chromosomal translocation, has been associated with increased expression of *ITGB1*, suggesting that RUNX1-ETO may target this gene in t(8;21) transformed cells (Ponnusamy *et al.* 2014). Furthermore, in

mouse studies, Runx1 and Runx3 were shown to regulate the expression of integrin genes Itga2b and Itgae, respectively (Grueter *et al.* 2005, Tanaka *et al.* 2012).

In addition, previously published microarray studies have shown altered expression of the integrin gene ITGB4, in individuals with leukaemia in which RUNX1 activity is altered. A study conducted by Valk *et al*. (2004) determined the gene expression profiles in samples of peripheral blood or bone marrow from 285 individuals with AML. In their analysis, they classified samples into groups based on their different molecular signatures, using cluster analyses. The different clusters of genes were found to be driven by the presence of chromosomal translocations such as t(8;21) and inv(16), as well as genetic mutations and oncogene expression (Valk *et al.* 2004). In the gene cluster which was driven by the presence of the t(8;21) chromosomal translocation, the integrin gene ITGB4 was significantly upregulated with a SAM score (assessing the minimal difference in gene expression compared to other AML samples) of 20.55 and a q-value of 0.14% (Valk *et al.* 2004). Additionally, a study by Ichikawa *et al*. (2006) determined the gene expression profiles of individuals with AML. The aim of this study was to identify genes whose dysregulation is associated with t(8;21) and inv(16) as well as genes with expression disrupted by both chromosomal translocations. Through this analysis, the ITGB4 gene was similarly identified to be significantly upregulated in the presence of the t(8;21) chromosomal translocation.

Furthermore, previous microarray studies have also shown altered expression of the ITGB5 gene in individuals with leukaemia with disrupted RUNX1. A study by Michaud *et al*. (2008) determined the gene expression profiles of cell lines with *RUNX1* mutations from individuals with FPD-AML and HeLa cells with *RUNX1* and *CBFβ* overexpressed. Through their analysis, *ITGB5* was found to be down-regulated in HeLa cells with *RUNX1* and *CBFβ* overexpressed, with a M-value (log2 of fold change) of -0.24 and -0.43 for each of the two biological replicates (Michaud *et al.* 2008). Additionally, another microarray study demonstrated that RUNX1, RUNX2 and RUNX3 transcription factors regulate a common set of genes involved in cell adhesion and survival (Wotton *et al.* 2008). In this study, *RUNX1*, *RUNX2* and *RUNX3* were overexpressed in NIH 3T3 fibroblast cells and changes in gene expression compared to control cells were measured. Through this analysis, *ITGB5* was found to be highly regulated by all three RUNX transcription factors and was up-regulated in fibroblast cells with RUNX1, RUNX2 and RUNX3 overexpressed, with fold changes of 1.68, 1.96 and 2.71, respectively (Wotton

et al. 2008). Furthermore, the study by Ichikawa *et al*. (2006) described above found altered expression of the ITGB5 gene to be associated with the inv(16) chromosomal translocation, suggesting that RUNX1 may be involved in the regulation of *ITGB5*, since CBFβ is required for RUNX1 function.

In support of these findings, a previous microarray study conducted by our research group also showed altered *ITGB4* and *ITGB5* expression in haematopoietic cells with RUNX1 expression disrupted. This microarray study analysed changes in gene expression when *RUNX1* was altered in Jurkat T cells to identify potential target genes. Novel RUNX1 target genes were identified according to altered gene expression profiles following down-regulation of *RUNX1* by transfection of Jurkat T-cells with RUNX1 siRNAs (Oakford and Holloway, unpublished). The knock-down of *RUNX1* in Jurkat T-cells resulted in altered expression of a large number of genes, including known RUNX1 target genes, such as *GM-CSF* and *IL-3*. In this dataset, integrin genes ITGB4 and ITGB5 were found to have significantly altered gene expression. Knock-down of *RUNX1* resulted in increased expression of *ITGB4* and *ITGB5*, with log2 fold changes of 1.623 and 0.957, respectively (Oakford and Holloway, unpublished).

Thus, analysis of previously published microarray studies, as well as a study conducted by our research group, identified the integrin genes, ITGB4 and ITGB5, as potential RUNX1 target genes due to their deregulation when RUNX1 or CBFβ was altered in haematopoietic cells. Interestingly, while RUNX1 generally functions as a transcriptional activator, its disruption consistently resulted in upregulation of *ITGB4*, while the effect on *ITGB5* was different depending on the cellular context. This suggests that investigation of the regulation of these genes by RUNX1 may provide important insight into the function of this transcription factor. In addition, although integrins have been known to play key roles in cell-cell interactions in both normal and disease processes, the regulation of integrin gene expression and the mechanisms contributing to their dysregulation remains largely unexplored. Therefore, an aim of this study was to investigate the regulation of integrin genes by RUNX1 in haematopoietic cells and particularly to determine if the integrin genes, ITGB4 and ITGB5, are directly regulated by RUNX1.

3.2 Results

3.2.1 Identification of Integrin Genes as Potential RUNX1 Targets

While *ITGB4* and *ITGB5* were identified as potential RUNX1 target genes through microarray studies, this type of analysis does not distinguish between direct and indirect targets. Therefore, publicly available ChIP-seq data investigating RUNX1 binding across the genome in haematopoietic cells were interrogated (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Tijssen *et al.* 2011). The BloodChIP database (http://www.med.unsw.edu.au/CRCWeb.nsf/page/BloodChIP; Chacon *et al.* 2014) was used to interrogate these ChIP-seq studies (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Tijssen *et al.* 2011). This database has assembled genome-wide binding profiles of seven key haematopoietic transcription factors (RUNX1, FLI1, ERG, GATA2, SCL, LYL1 and LMO2) in human CD34+ haematopoietic stem/progenitor cells (GSE45144), megakaryocytes (GSE24674), the SKNO-1 AML cell line which is positive for the t(8;21) chromosomal translocation (GSE23730), and the K562 CML cell line (GSE24779, GSE29196 and GSE31477). The database is designed to enable researchers to easily interrogate and visualise data from these studies. The database also contains information on genome-wide gene expression in the different cell lines (GSE30029, GSE34594 and GSE28135), as well as histone profiles (GSE18927 and GSE29611) and the presence of DNase I hypersensitive sites (GSM646567). Data from two further ChIP-seq studies examining RUNX1 binding became available during the course of this study (Ptasinska *et al.* 2014, Trombly *et al.* 2015). These studies were performed in Kasumi-1 cells with an ETO and/or RUNX1 antibody, therefore representing RUNX1-ETO and/or RUNX1 binding, and was downloaded and visualised using the UCSC Genome Browser (Kent *et al.* 2002).

Firstly, to examine the quality of the available ChIP-seq data, integrin genes ITGAL and ITGA2B previously identified to be regulated by RUNX1 in human and mouse cells, respectively (Puig-Kröger *et al.* 2000, Puig-Kröger *et al.* 2003, Tanaka *et al.* 2012), were interrogated for RUNX1/RUNX1-ETO binding at their promoter regions in these datasets. A region spanning from -1000 bp to +300 bp of the transcription start site is typically examined when interrogating regions surrounding the transcription start site for regulatory elements and was therefore analysed in this study. Promoters generally encompass approximately 250 bp upstream of the transcription start site and can span to

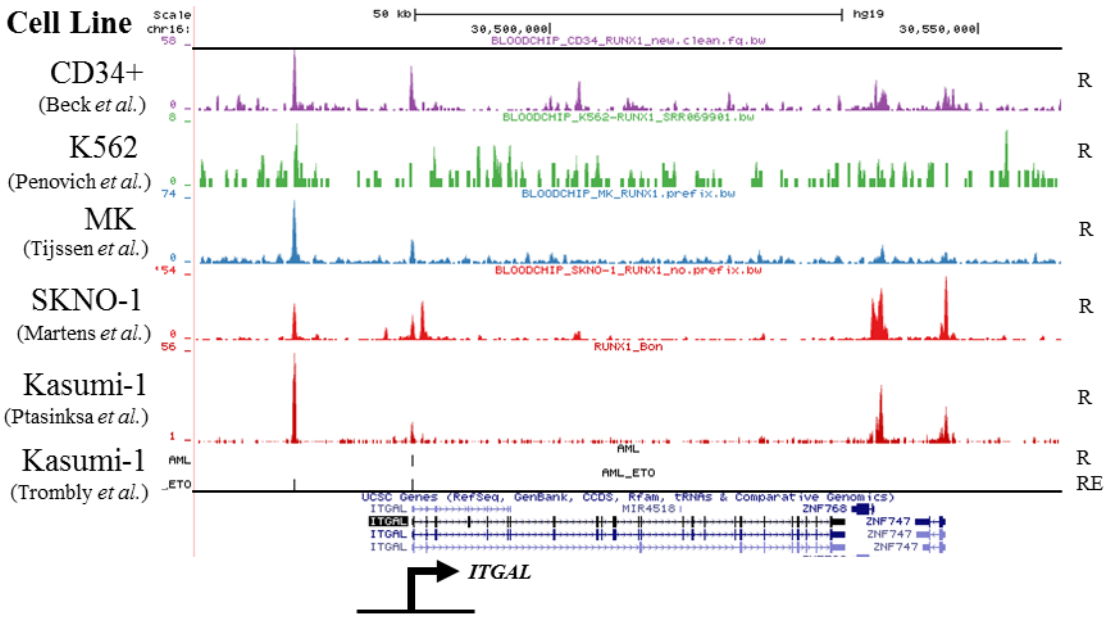
250 bp downstream (Butler and Kadonaga 2002, Maston *et al.* 2006). As expected, RUNX1 and RUNX1-ETO were detected at promoter regions of both *ITGAL* and *ITGA2B* in CD34+ cells (Beck *et al.* 2013), megakaryocytes (Tijssen *et al.* 2011), SKNO-1 cells (Martens *et al.* 2012) and Kasumi-1 cells (Ptasinska *et al.* 2014, Trombly *et al.* 2015), thus providing confidence in the quality of these data (Figure 3.1). However, specific RUNX1 binding was not detected at these integrin promoter regions in K562 cells (Pencovich *et al.* 2011), which may be due to lower levels of RUNX1 in these cells (Figure 3.1). The data generated in K562 cells may therefore be less reliable for detecting RUNX1 binding, and this is also supported by the absence of RUNX1 at the well-described target genes GM-CSF and M-CSF (data not shown).

Since binding of RUNX1/RUNX1-ETO was detected at the promoters of the known target genes ITGAL and ITGA2B, these data were further interrogated for RUNX1/RUNX1-ETO binding at promoter regions of *ITGB4* and *ITGB5*. While peaks can be visualised across the ITGB4 and ITGB5 genes in all the ChIP-seq studies, the only peak 'called' at both of the integrin promoters was in the SKNO-1 cell dataset (Figure 3.2). The peak calling used in this analysis was obtained from the original studies (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Tijssen *et al.* 2011, Trombly *et al.* 2015). Trombly *et al.* (2015) used the peak calling program MACS with default settings, using a *p* value of $<10^{-20}$, while data in the BloodChIP database (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Tijssen *et al.* 2011) were interrogated using HOMER, MACS and Partek, and peaks were included if they were called by 2 or more of the 3 programs. Therefore, whether a peak is 'called' or not is dependent on the thresholds set in the analysis, but the peaks that could be visualised in the other datasets were relatively small and therefore potentially represent background. Regardless, the presence of RUNX1 at the ITGB4 and ITGB5 promoters in SKNO-1 cells suggests that it has the potential to regulate these integrin genes.

Integrin receptors are formed by the binding of α and β subunits, which are encoded by separate genes. The β4 subunit produced by the ITGB4 gene heterodimerises with the α6 subunit, encoded by the ITGA6 gene. While the β4 subunit heterodimerises only with α6, the α6 subunit can also heterodimerise with the β1 subunit, encoded by the ITGB1 gene. Similar to the β4 subunit, the β5 subunit, encoded by *ITGB5*, only heterodimerises with one subunit, αV, which is encoded by the ITGAV gene. To determine whether RUNX1 and RUNX1-ETO may also target the genes encoding these binding partners, which

would enable co-regulation of these genes, *ITGA6*, *ITGB1* and *ITGAV* were also examined for binding of these transcription factors. Interestingly, RUNX1 was detected at the ITGA6 promoter in megakaryocytes, SKNO-1 cells and Kasumi-1 cells (Figure 3.3A), while RUNX1-ETO was detected at the ITGA6 promoter in Kasumi-1 cells (Figure 3.3 and 3.4). RUNX1 was also detected at the ITGB1 and ITGAV promoters in CD34+, SKNO-1 cells and Kasumi-1 cells (Figure 3.3B and 3.4), Together, these data suggest that RUNX1 may regulate integrin genes ITGA6, ITGB1 and ITGAV, in addition to ITGB4 and ITGB5.

A



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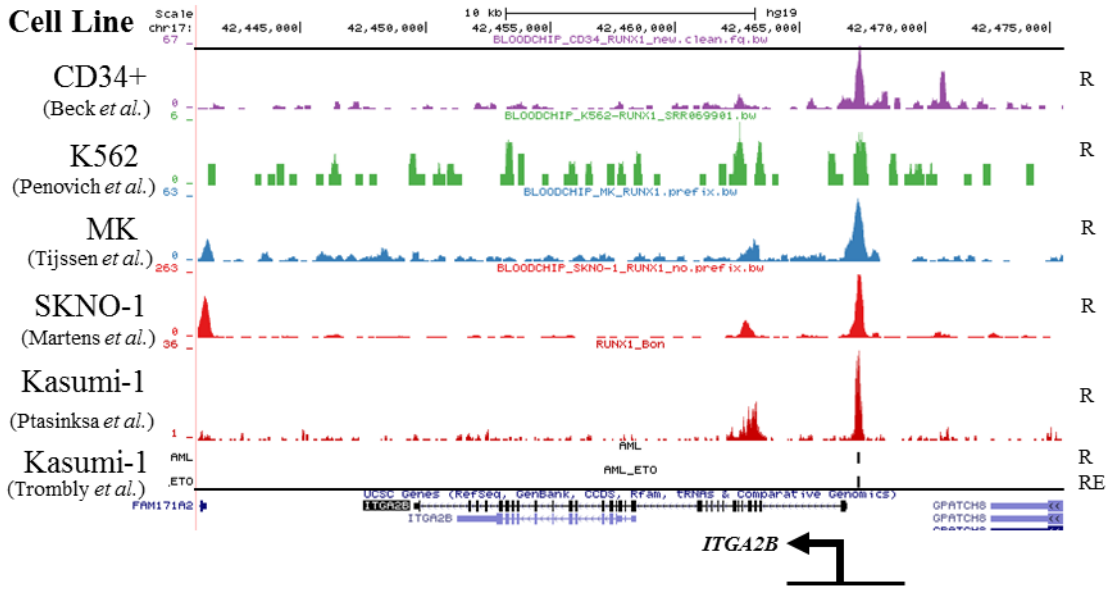
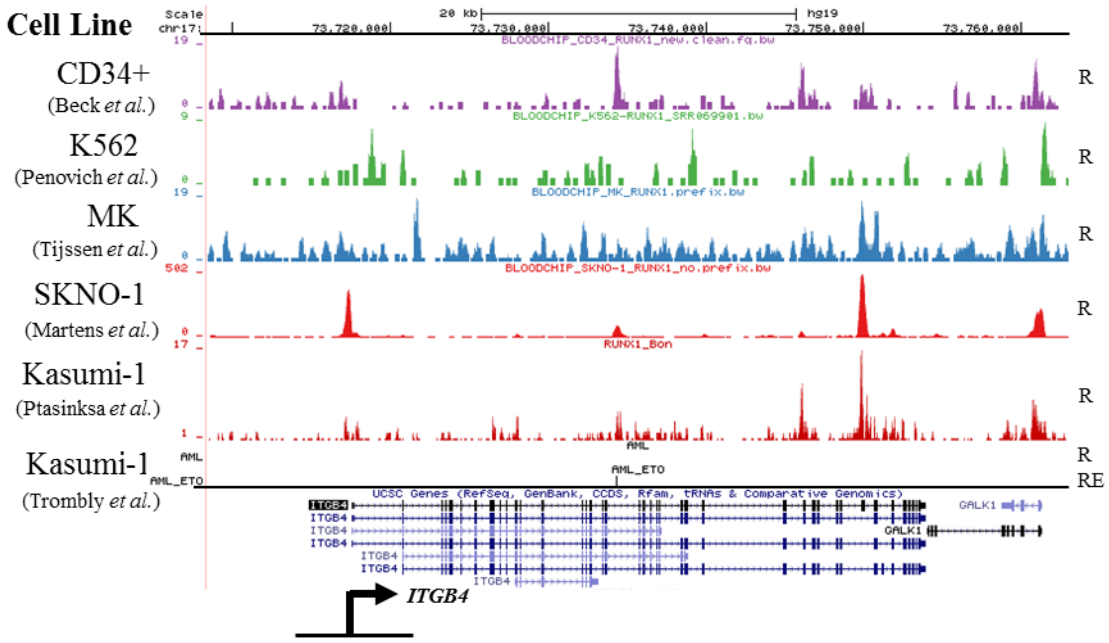


Figure 3.1 - RUNX1 and RUNX1-ETO binding of *ITGAL* and *ITGA2B* in haematopoietic cells. Data from ChIP-seq studies (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Ptasinska *et al.* 2014, Tijssen *et al.* 2011, Trombly *et al.* 2015) were downloaded and visualised using the UCSC Genome Browser (Kent *et al.* 2002). Screen shots were taken from the UCSC browser and show RUNX1 (R) and RUNX1-ETO (RE) binding across **(A)** ITGAL and **(B)** ITGA2B integrin genes in different cell types. Peaks and lines represent RUNX1/RUNX1-ETO binding. The transcription start site for each integrin gene is shown at the bottom of the figure by a black right-angled arrow. The integrin gene is represented at the bottom of the figure with exons shown as bars. The major transcript is in black.

A



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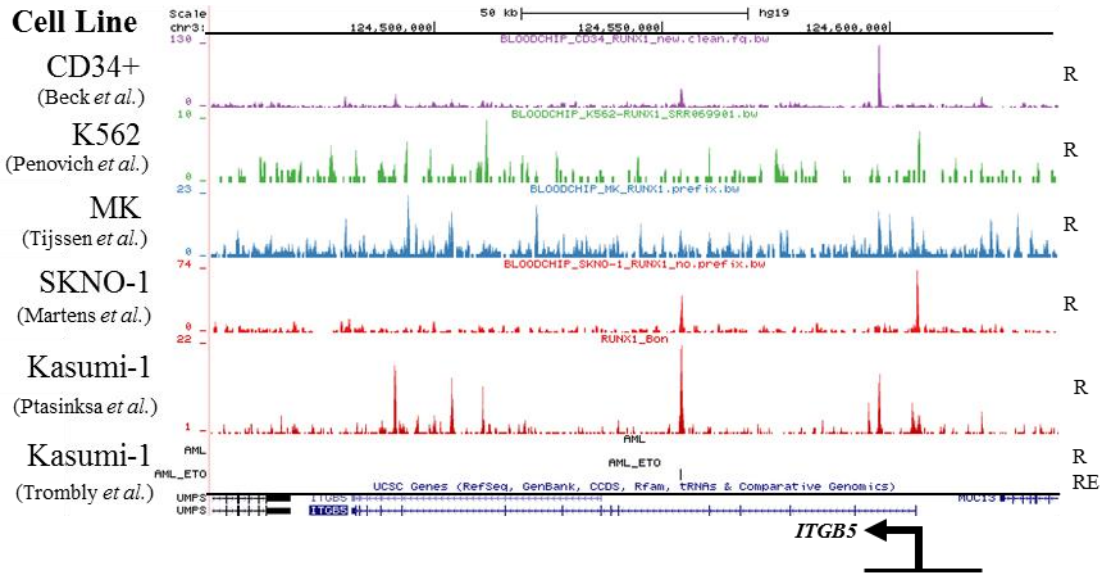
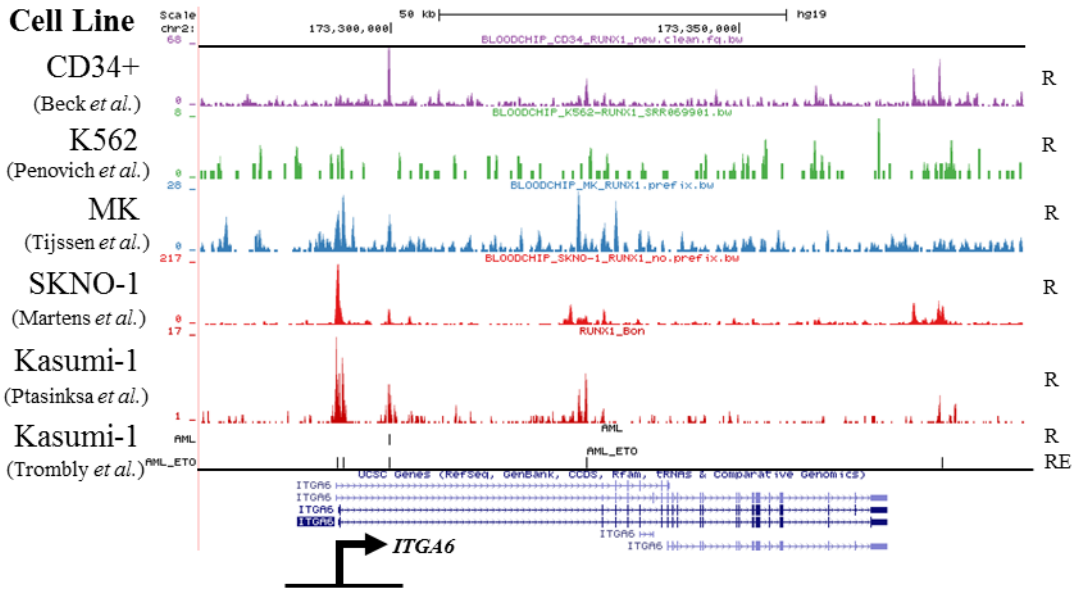


Figure 3.2 - RUNX1 and RUNX1-ETO binding of *ITGB4* and *ITGB5* in haematopoietic cells. Data from ChIP-seq studies (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Ptasinska *et al.* 2014, Tijssen *et al.* 2011, Trombly *et al.* 2015) were downloaded and visualised using the UCSC Genome Browser (Kent *et al.* 2002). Screen shots were taken from the UCSC browser and show RUNX1 (R) and RUNX1-ETO (RE) binding across **(A)** ITGB4 and **(B)** ITGB5 integrin genes in different cell types. Peaks and lines represent RUNX1/RUNX1-ETO binding. The transcription start site for each integrin gene is shown at the bottom of the figure by a black right-angled arrow. The integrin gene is represented at the bottom of the figure with exons shown as bars. The major transcript is in black/dark blue.

A



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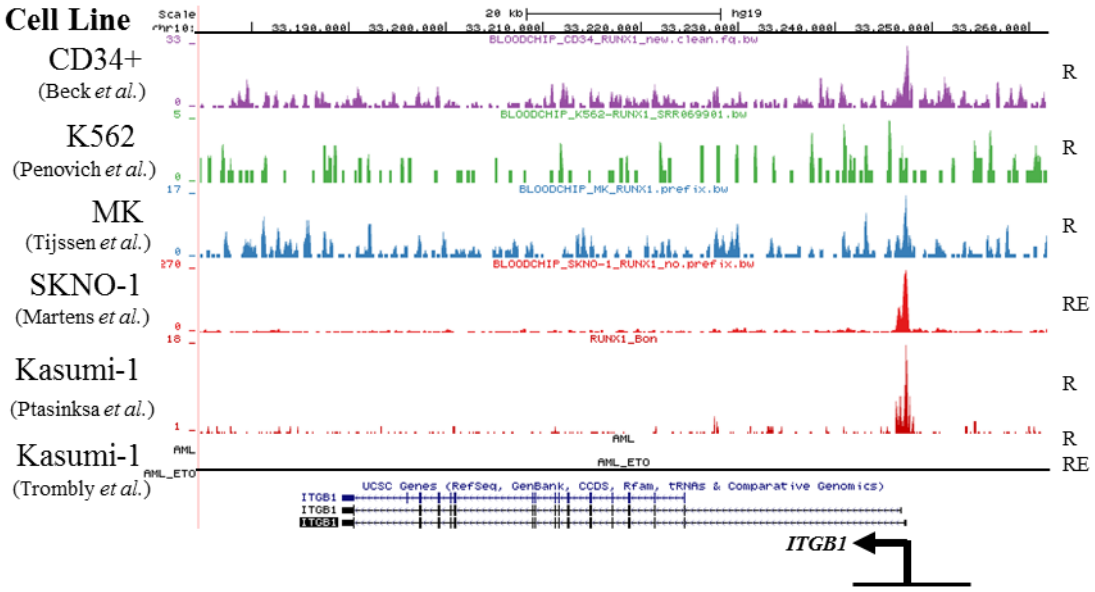


Figure 3.3 - RUNX1 and RUNX1-ETO binding of *ITGA6* and *ITGB1* in haematopoietic cells. Data from ChIP-seq studies (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Ptasinska *et al.* 2014, Tijssen *et al.* 2011, Trombly *et al.* 2015) were downloaded and visualised using the UCSC Genome Browser (Kent *et al.* 2002). Screen shots were taken from the UCSC browser and show RUNX1 (R) and RUNX1-ETO (RE) binding across **(A)** ITGA6 and **(B)** ITGB1 integrin genes in different cell types. Peaks and lines represent RUNX1/RUNX1-ETO binding. The transcription start site for each integrin gene is shown at the bottom of the figure by a black right-angled arrow. The integrin gene is represented at the bottom of the figure with exons shown as bars. The major transcript is in black/dark blue.

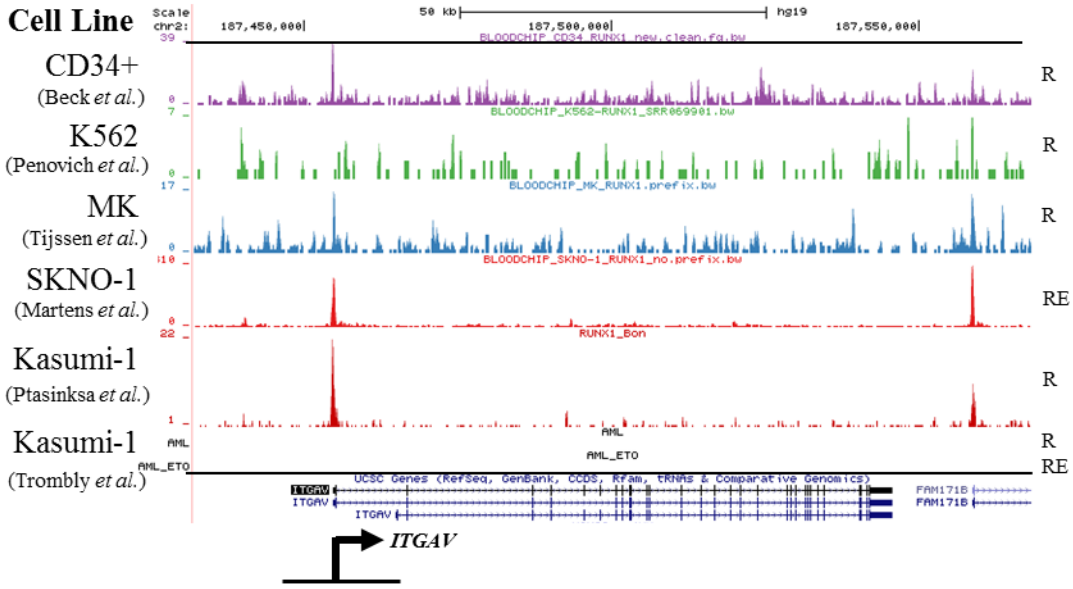


Figure 3.4 - RUNX1 and RUNX1-ETO binding of *ITGAV* in haematopoietic cells. Data from ChIP-seq studies (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Ptasinska *et al.* 2014, Tijssen *et al.* 2011, Trombly *et al.* 2015) were downloaded and visualised using the UCSC Genome Browser (Kent *et al.* 2002). Screen shots were taken from the UCSC browser and show RUNX1 (R) and RUNX1-ETO (RE) binding across ITGAV integrin gene in different cell types. Peaks and lines represent RUNX1/RUNX1-ETO binding. The transcription start site for *ITGAV* is shown at the bottom of the figure by a black right-angled arrow. The integrin gene is represented at the bottom of the figure with exons shown as bars. The major transcript is in black.

3.2.2 Integrin Expression and Response to PMA in Leukaemic Cell Lines

3.2.2.1 Integrin Expression in Leukaemic Cell Lines

Based on the analysis outlined above, the integrin genes ITGB4, ITGB5, ITGA6, ITGAV and ITGB1 were identified as potential targets of RUNX1/RUNX1-ETO in haematopoietic cells. To determine whether these integrin genes are expressed in haematopoietic cells, mRNA levels were analysed in K562, KG-1a and Kasumi-1 myeloid cell lines using RT-qPCR. The K562 cell line is derived from an individual with CML and resemble undifferentiated granulocytes and erythrocytes (Lozzio and Lozzio 1975); the KG-1a cell line, which are promyeloblasts, is derived from an individual with AML and resemble myeloblasts (Koeffler *et al.* 1980); and the Kasumi-1 cell line is derived from an individual with AML and resemble myeloblasts (Asou *et al.* 1991). The K562 cell line was chosen for analysis as it represents cells that are more differentiated than the other cell lines and the Kasumi-1 cell line was included in the analysis because it possesses the t(8;21) chromosomal translocation and therefore produces the RUNX1-ETO fusion protein.

RNA was isolated from the cell lines and analysed by RT-qPCR for *ITGB4*, *ITGB5*, *ITGA6*, *ITGAV* and *ITGB1* expression, as well as the expression of *RUNX1* and *RUNX1-ETO*. To distinguish between RUNX1 and RUNX1-ETO mRNA, specific primers were used that bind to an exon that is only present in RUNX1, while primers which span the RUNX1-ETO junction were used to specifically detect this transcript. All mRNA levels were normalised to GAPDH mRNA levels to account for discrepancies in cDNA synthesis and RNA input. RUNX1 mRNA was detected in all three leukaemic cell lines although at significantly higher levels in Kasumi-1 cells. As expected, RUNX1-ETO mRNA was only detected in the Kasumi-1 cell line (Figure 3.5). Analysis of integrin mRNA levels determined that the integrins are differentially expressed across the cell lines. The ITGB4 gene is expressed at higher levels in the Kasumi-1 cell line, very low levels in K562 cells, and was not detected in the KG-1a cell line (Figure 3.6A). In contrast, the ITGA6 gene is expressed in both KG-1a and Kasumi-1 cell lines but expressed at very low levels in the K562 cell line (Figure 3.6A). The ITGB1 gene is expressed in all cell lines, although at higher levels in Kasumi-1 cells (Figure 3.6A). *ITGB5* is expressed at higher levels in the K562 cell line and at relatively low levels in the KG-1a and Kasumi-1 cell lines (Figure 3.6B), while ITGAV mRNA was detected in all cell lines examined (Figure 3.6B). This expression data is in keeping with the data

available through the BloodChIP database which shows ITGB4 and ITGA6 expressed at lower levels in K562 cells (GSE28135), compared to ITGB1 and ITGB5 which are expressed at higher levels (data not shown). Additionally, ITGB4, ITGA6, ITGB1 and ITGB5 mRNA was detected in SKNO-1 cells, which like Kasumi-1 cells are positive for the t(8;21) chromosomal translocation (data not shown).

Based on the integrin mRNA levels detected by RT-qPCR, it is likely that the myeloid cell lines express different integrin receptors on their surface. The Kasumi-1 cell line may express both the integrin receptors α6β4 and α6β1, whereas the KG-1a cell line is likely to only express the integrin receptor α6β1 due to low levels of ITGB4 gene expression. In contrast, the data suggests that the K562 cell line does not have the capacity to express either α6β4 or α6β1, due to low levels of both ITGB4 and ITGA6 mRNA, but different β1 integrin receptors may be expressed by these cells due to the presence of ITGB1 mRNA. The results also suggest that only the K562 cell line is likely to express the αVβ5 receptor due to high levels of ITGB5 and ITGAV mRNA detected, whereas the KG-1a and Kasumi-1 cells are less likely to express the αVβ5 receptor, due to low levels of ITGB5 mRNA, but may express a different αV receptor, such as αVβ3, αVβ6 or αVβ8.

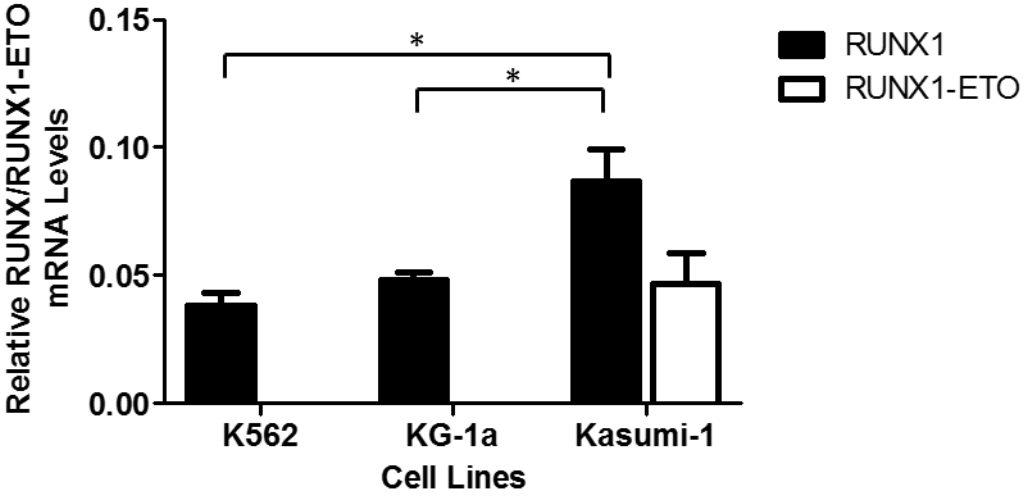
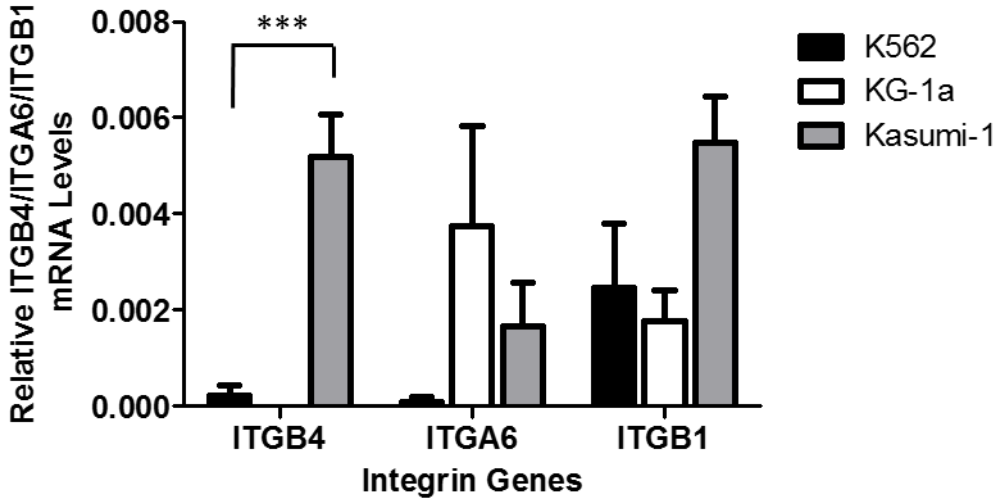


Figure 3.5 – RUNX1 and RUNX1-ETO mRNA levels in leukaemic cell lines. Total mRNA was isolated from K562, KG-1a and Kasumi-1 cell lines, reversed transcribed and RUNX1 and RUNX1-ETO mRNA levels were analysed using RT-qPCR. mRNA levels were normalised to GAPDH. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using one-way ANOVA, Newman-Keuls Multiple Comparison Test, *p<0.05.

A



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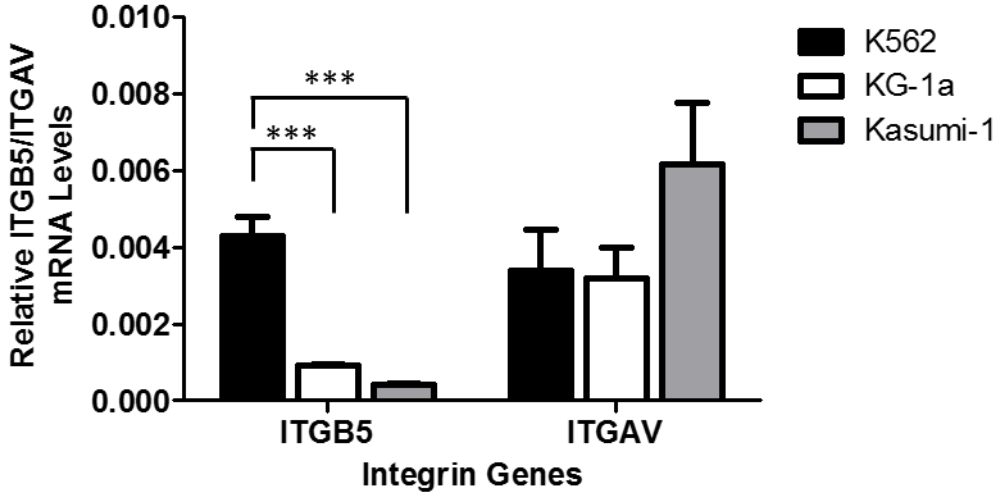


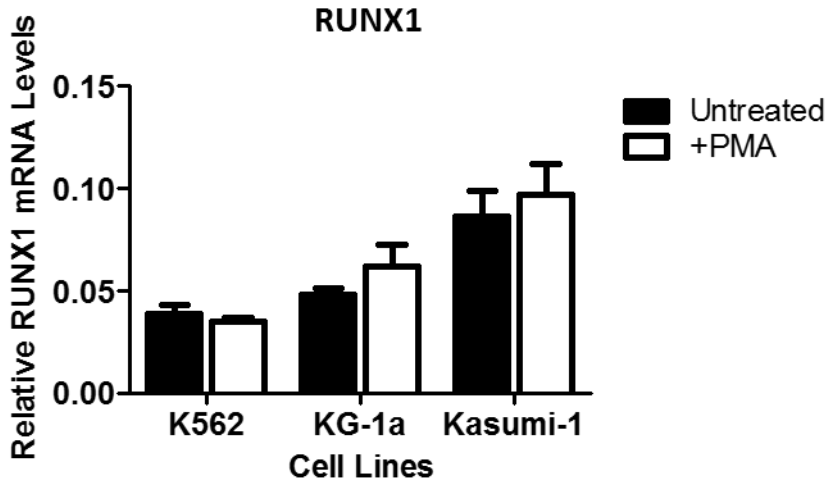
Figure 3.6 – Integrin mRNA levels in leukaemic cell lines. Total mRNA was isolated from K562, KG-1a and Kasumi-1 cell lines, reversed transcribed and **(A)** ITGB4, ITGA6 and ITGB1, and **(B)** ITGB5 and ITGAV mRNA levels were analysed using RT-qPCR. mRNA levels were normalised to GAPDH. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using one-way ANOVA, Newman-Keuls Multiple Comparison Test, ***p<0.001.

3.2.2.2 Effect of PMA on Integrin Expression in Myeloid Cell Lines

Myelogenous leukaemia cell lines, such as K562, KG-1a and Kasumi-1, can be induced to differentiate in response to treatment with agents such as phorbol esters. Treatment of myelogenous leukaemia cell lines with phorbol esters results in increased adhesion and increased number of phagocytic cells due to differentiation of the cell lines (Koeffler *et al.* 1980, Pegoraro *et al.* 1980). Since integrins play a critical role in cell adhesion, the functional and morphological changes observed when myelogenous leukaemia cells are treated with phorbol esters may be at least partly attributed to changes in integrin expression. Therefore, *ITGB4*, *ITGA6*, *ITGB1*, *ITGB5* and *ITGAV* gene expression was analysed in K562, KG-1a and Kasumi-1 leukaemic cell lines following treatment with the phorbol ester, PMA.

The leukaemic cell lines were treated with PMA for 72 hours and changes in morphology and adhesion were monitored using microscopy. Percentage of flattened/adherent cells was determined by visually observing the cells and counting the flattened cells in several fields of view. Expression of the integrin genes as well as *RUNX1* and *RUNX1-ETO* was determined by RT-qPCR. Treatment with PMA resulted in changes in morphology, specifically flattening and increased adherence of all cell lines (data not shown). The Kasumi-1 cells displayed the most flattened/adherent morphology, while KG-1a cells only displayed minimal changes in morphology and adherence. Treatment with PMA resulted in no change in RUNX1 mRNA levels in all three cell lines, and there was also no change in RUNX1-ETO mRNA levels in the Kasumi-1 cell line (Figure 3.7). In contrast, expression of all integrin genes examined increased in K562 cells following PMA treatment, with a significant increase detected for *ITGB4*, *ITGB5* and *ITGAV* (Figure 3.8 and 3.9). In contrast to K562 cells, no significant change in expression of any of the integrins examined was detected in KG-1a and Kasumi-1 cells following treatment with PMA (Figure 3.8 and 3.9). Together, these data suggest that increased adhesion of the K562 PMA treated cells may be in part due to increased expression of the integrins examined here. Although Kasumi-1 treated cells displayed the most flattened/adherent phenotype of the cell types examined, this increased adhesion is likely due to altered expression of other integrins or cell adhesion molecules, that were not examined here.

A



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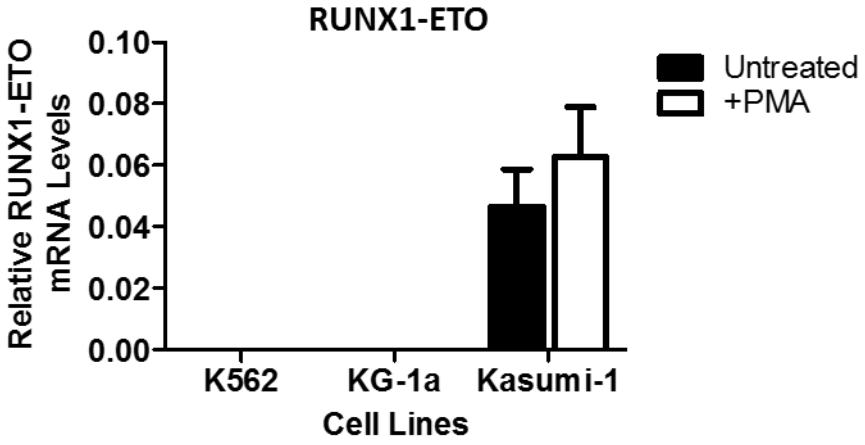

Figure 3.7 – RUNX1 and RUNX1-ETO mRNA levels in PMA treated leukaemic cell lines. Total mRNA was isolated from untreated and PMA treated K562, KG-1a and Kasumi-1 cell lines, reversed transcribed and **(A)** RUNX1 and **(B)** RUNX1-ETO mRNA levels were analysed using RT-qPCR. mRNA levels were normalised to GAPDH. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using Students' *t* Test, with no significant difference detected between untreated and PMA treated cells.

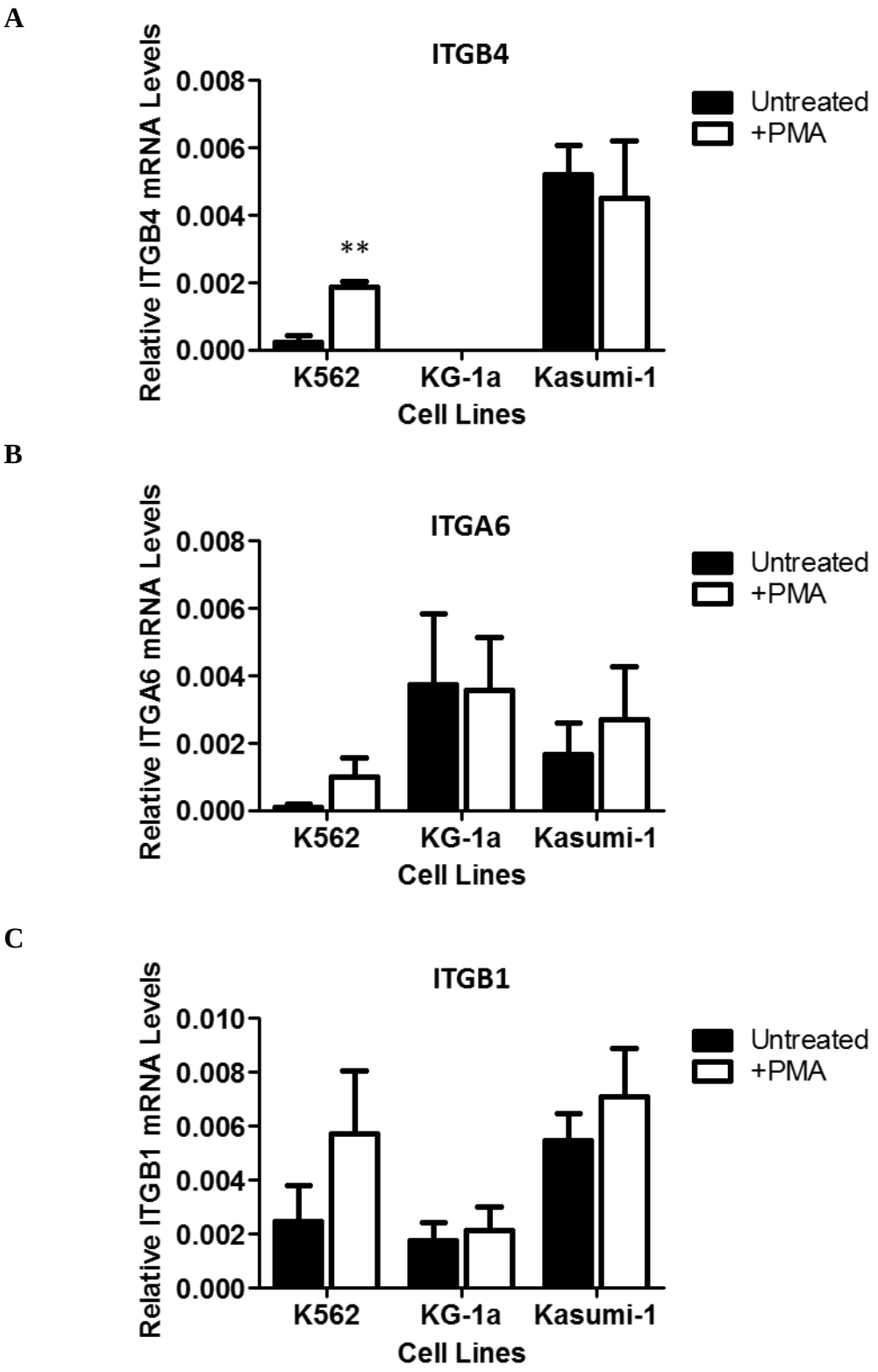

Figure 3.8 – ITGB4, ITGA6 and ITGB1 mRNA levels in PMA treated leukaemic cell lines. Total mRNA was isolated from untreated and treated K562, KG-1a and Kasumi-1 cell lines, reversed transcribed and **(A)** ITGB4, **(B)** ITGA6 and **(C)** ITGB1 mRNA levels were analysed using RT-qPCR. mRNA levels were normalised to GAPDH. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using Students' *t* Test, $^{**}p<0.01$.

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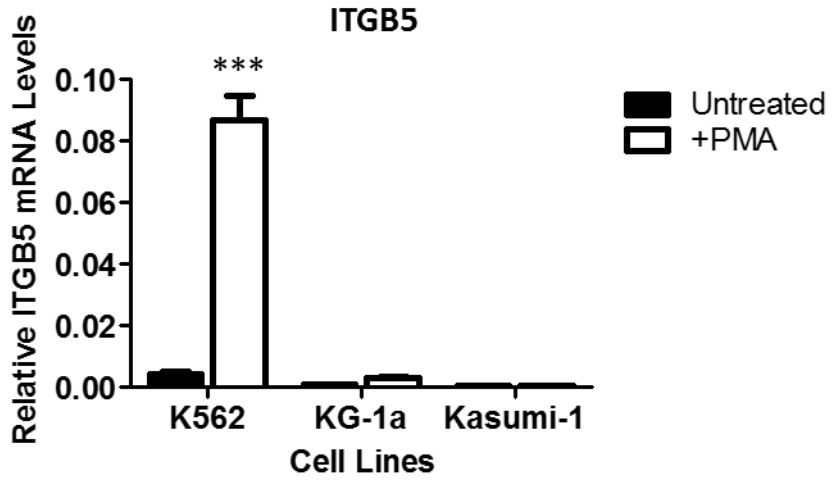

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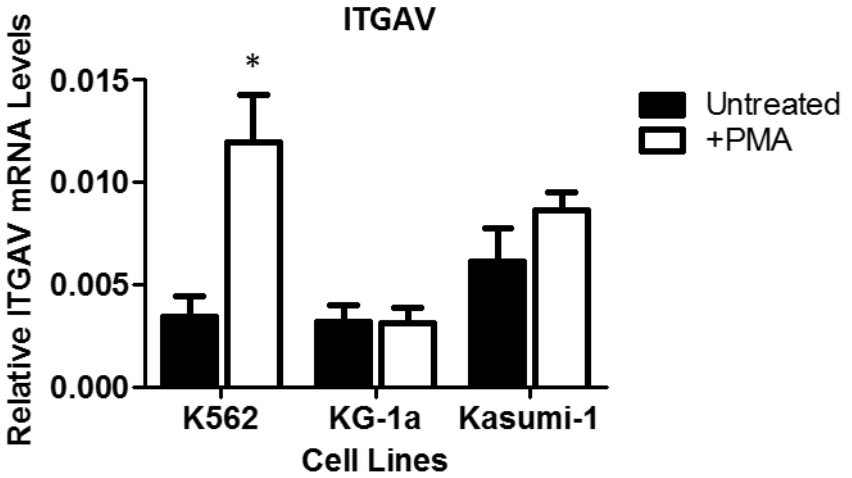


Figure 3.9 – ITGB5 and ITGAV mRNA levels in PMA treated leukaemic cell lines. Total mRNA was isolated from non-activated and activated K562, KG-1a and Kasumi-1 cell lines, reversed transcribed and **(A)** ITGB5 and **(B)** ITGAV mRNA levels were analysed using RT-qPCR. mRNA levels were normalised to GAPDH. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using Students' *t* Test, ***$p<0.001$, *$p<0.05$.

3.2.3 Regulation of Integrin Promoters by RUNX1

Analysis of RUNX1/RUNX1-ETO genome localisation data (Section 3.2.1) identified RUNX1 and RUNX1-ETO bound to the promoters of *ITGB4*, *ITGA6*, *ITGB1*, *ITGB5* and *ITGAV* in haematopoietic cells. Furthermore, these integrin genes were found to be expressed, albeit at different levels, in myeloid cell lines (Section 3.2.2). Therefore, to investigate the potential RUNX1 regulation of these integrin genes in haematopoietic cells further, integrin promoter regions were analysed bioinformatically for RUNX binding motifs and RUNX1 regulation of integrin promoters was analysed using reporter assays. Promoter regions of each integrin gene examined for RUNX binding sites and used in reporter assays was determined based on the ChIP-seq analysis. Regions incorporating the DNA sequence corresponding to RUNX1 peaks in the ChIP data analysed in *Section 3.2.1* were examined in reporter assays, and the presence of RUNX binding motifs was investigated within those regions.

The presence of RUNX binding motifs was determined using the MatInspector tool in the Genomatix bioinformatics suite (http://www.genomatix.de/). MatInspector identifies potential transcription factor binding sites within a query sequence. The stringency of this tool can be altered to determine higher or lower affinity binding sites by altering core or matrix similarity values. The core similarity value represents the most conserved bases within a RUNX binding motif and is pre-set to consider 4 bases of a potential RUNX site. A core similarity value of 1 is given when there are no mismatches in the core sequence. The core sequence of RUNX binding sites therefore can be any 4 bases which match the TGT/cGGT binding motif. The matrix similarity value analyses the bases adjacent to the core sequence and determines the frequency of the most conserved nucleotide at a particular position relative to the core sequence. A matrix similarity of 0.80 or greater is considered as a good match for a binding motif. Therefore, analysis of core and matrix similarity values allows the evaluation of potential binding affinity for a particular site. Taken together, RUNX binding motifs with high binding affinities considered in this study had minimum values of 0.90 for core similarity and 0.75 for matrix similarity. Using these settings, at least one RUNX motif was identified within the promoter regions of all integrin genes examined (Table 3.1).

Integrin	Position	Core Similarity	Matrix Similarity	Strand	Sequence
ITGB4	-1096 to -1110	1	0.807	(+)	catgGTGGttcacgc
	-976 to -990	1	0.808	(+)	gggcGTGGtggcggg
	-884 to -898	1	0.802	(-)	tgcaGTGGtgcaatc
	-773 to -787	1	0.847	(+)	acccGTGGtaagcag
	-370 to -384	1	0.822	(-)	ggcTGTGctgctgct
	-190 to -204	1	0.759	(+)	agcgGTGGctcctcc
	-132 to -146	0.909	0.8	(-)	ggctGCGGctcccgc
ITGA6	-644 to -658	1	0.806	(-)	gctTGTGgaagaagt
	-473 to -487	1	0.834	(+)	cgcTGTGatcattt
	-335 to -349	1	0.813	(-)	agatGTGGgcccacg
	-240 to -254	1	0.815	(+)	ctcTGTGctactcgg
	-229 to -243	1	0.973	(-)	aatTGTGgttgccga
	-217 to -231	1	0.86	(-)	ctctGTGGacagaat
	+10 to +24	0.909	0.786	(+)	ttcaGCGGtcgcgag
	+110 to +124	0.909	0.904	(+)	ggctGCGGtagcagc
	+219 to +233	1	0.857	(+)	agcTGTGcttgctct
ITGB1	-454 to -468	1	0.771	(-)	ccacGTGGtccttct
	-447 to -461	1	0.873	(+)	ccacGTGGtttttgg
	+240 to +254	1	0.821	(+)	gggtGTGGgagcgcg
ITGB5	-420 to -434	0.909	0.75	(-)	aaagGCGGttgctag
	-318 to -332	1	0.887	(+)	ctcaGTGGttcgag
	-169 to -183	1	0.833	(+)	ccgtGTGGcggccgg
ITGAV	-94 to -108	1	0.829	(-)	tgctGTGGagctgga

Table 3.1 – RUNX binding motifs identified in integrin promoter regions. Binding motifs of the RUNX transcription factors were identified in integrin promoter regions using the MatInspector tool from the Genomatix bioinformatics suite (http://www.genomatix.de/). The locations of the motifs are defined relative to the transcription start site. The core and matrix similarity values indicate the potential binding affinities of each site. The RUNX binding motifs are shown in red and the core sequences identified by MatInspector are shown in captial letters. RUNX binding motifs located on the sense strand are denoted with a (+) while motifs located on the antisense strand are denoted with a (-).

RUNX1 and RUNX1-ETO effects on the integrin promoters were analysed using luciferase reporter assays. Integrin 5' UTRs, depicted schematically in *Figure 3.10*, were cloned adjacent to a luciferase gene in the pXPG plasmid, as detailed in *Chapter 2, Section 2.2*. Regions analysed incorporated the region identified as binding RUNX1 in ChIP data analysed in *Section 3.2.1* (Martens *et al.* 2012). The integrin reporter constructs were transfected into K562 myeloid cells along with RUNX1 or RUNX1-ETO expression plasmids. Cells were harvested 24 hours after transfection and luciferase activity was measured. To ensure that RUNX1 and RUNX1-ETO were successfully overexpressed in the K562 cells, protein levels were analysed in transfected cells using Western Blot analysis. Protein from K562 cells, was isolated 24 hours post transfection and RUNX1/RUNX1-ETO proteins were detected using RUNX1 and ETO specific antibodies. As a positive control, RUNX1 and RUNX1-ETO overexpression was also monitored in the Cos-7 cell line. Western Blot analysis shown in *Figure 3.11* demonstrates that both RUNX1 and RUNX1-ETO proteins were successfully overexpressed in K562 and Cos-7 cells.

Firstly, to determine the basal level of activity of the integrin reporter constructs, cells were transfected with the constructs alone and monitored for luciferase activity. Changes in luciferase activity with RUNX1 and RUNX1-ETO overexpressed were therefore compared to the basal level of activity of the reporter. Transfection of K562 cells with the RUNX1 expression plasmid resulted in increased luciferase activity for all integrin reporters except for ITGB1, with a significant increase detected for both ITGB4 and ITGA6 (Figure 3.12).

While RUNX1 activated the ITGA6 promoter, overexpression of RUNX1-ETO repressed the ITGA6 reporter (Figure 3.12B). In contrast, RUNX1-ETO did not appear to have any effect on the ITGB4 or ITGAV promoters (Figure 3.12A and 3.12E). However, on further analysis, RUNX1-ETO was shown to repress RUNX1 activation of the ITGB4 promoter in a dose-dependent manner, as demonstrated in *Figure 3.13*. Interestingly, while RUNX1 did not have an effect on the ITGB1 reporter, overexpression of RUNX1-ETO resulted in a significant decrease in activity (Figure 3.12C). In contrast to the other integrins, overexpression of RUNX1-ETO was shown to increase the activity of the ITGB5 reporter in a similar fashion to RUNX1 (Figure 3.12D).

Taken together, these results suggest that RUNX1 activates the ITGB4 and ITGA6 promoters and that RUNX1-ETO can repress both ITGB4 and ITGA6 promoters, competing with RUNX1 to block RUNX1 activation of the promoters. While RUNX1 increased activity of the ITGB5 and ITGAV promoters, this was not statistically significant. Additionally, RUNX1-ETO can activate the ITGB5 promoter to similar levels as to RUNX1 and can repress the ITGB1 promoter.

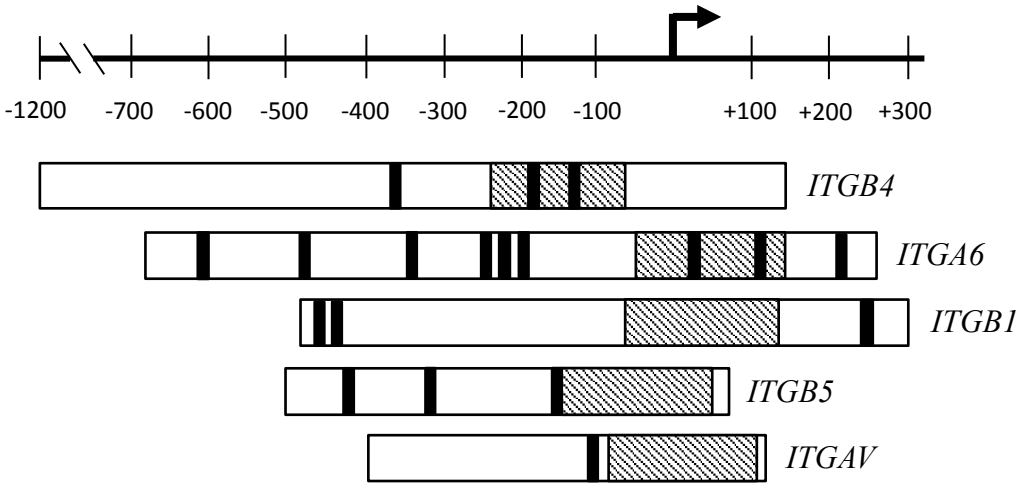
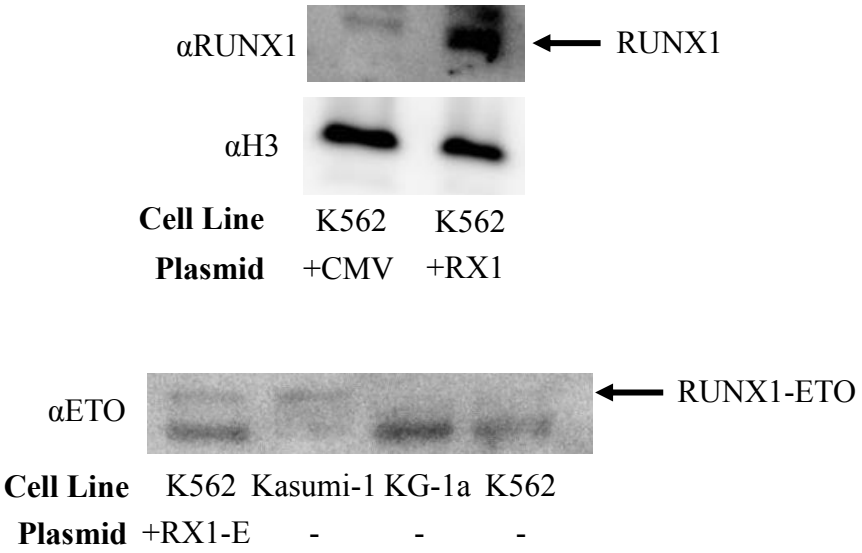


Figure 3.10 – Integrin promoter constructs. Schematic representation of integrin promoter regions analysed in reporter assays. RUNX motifs identified by MatInspector (http://www.genomatix.de/) are denoted by the small black rectangles (with a further 4 within the -700 to -1200 region of *ITGB4* not depicted). The boxes with diagonal lines correspond to the DNA sequence bound by RUNX1 in SKNO-1 cells in the ChIP-seq study by Martens *et al* (2012), as depicted in *Figures 3.1-3.4*. Primers were designed to amplify the depicted regions of ITGB4, ITGA6, ITGB1, ITGB5 and ITGAV integrin genes. These regions were cloned into the pXPG plasmid containing a luciferase gene for reporter assays. The scale indicates base pairs relative to the transcription start site, which is represented by the arrow.

A



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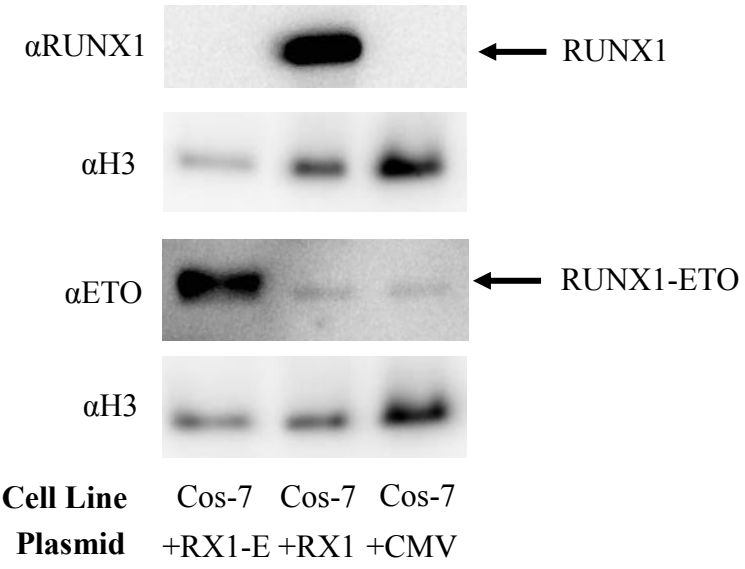


Figure 3.11 – Overexpression of RUNX1 and RUNX1-ETO in cell lines. Western blot analysis was used to examine RUNX1 and RUNX1-ETO expression in nuclear extracts prepared from **(A)** myeloid cell lines or **(B)** Cos-7 cells either untransfected or transfected with control plasmid (CMV), RUNX1 expression plasmid (RX1) or RUNX1-ETO expression plasmid (RX1-E). Nuclear extracts were subjected to Western Blot analysis with the indicated antibodies used. Arrows indicate RUNX1 and RUNX-ETO protein bands. Histone H3 was monitored as a loading control.

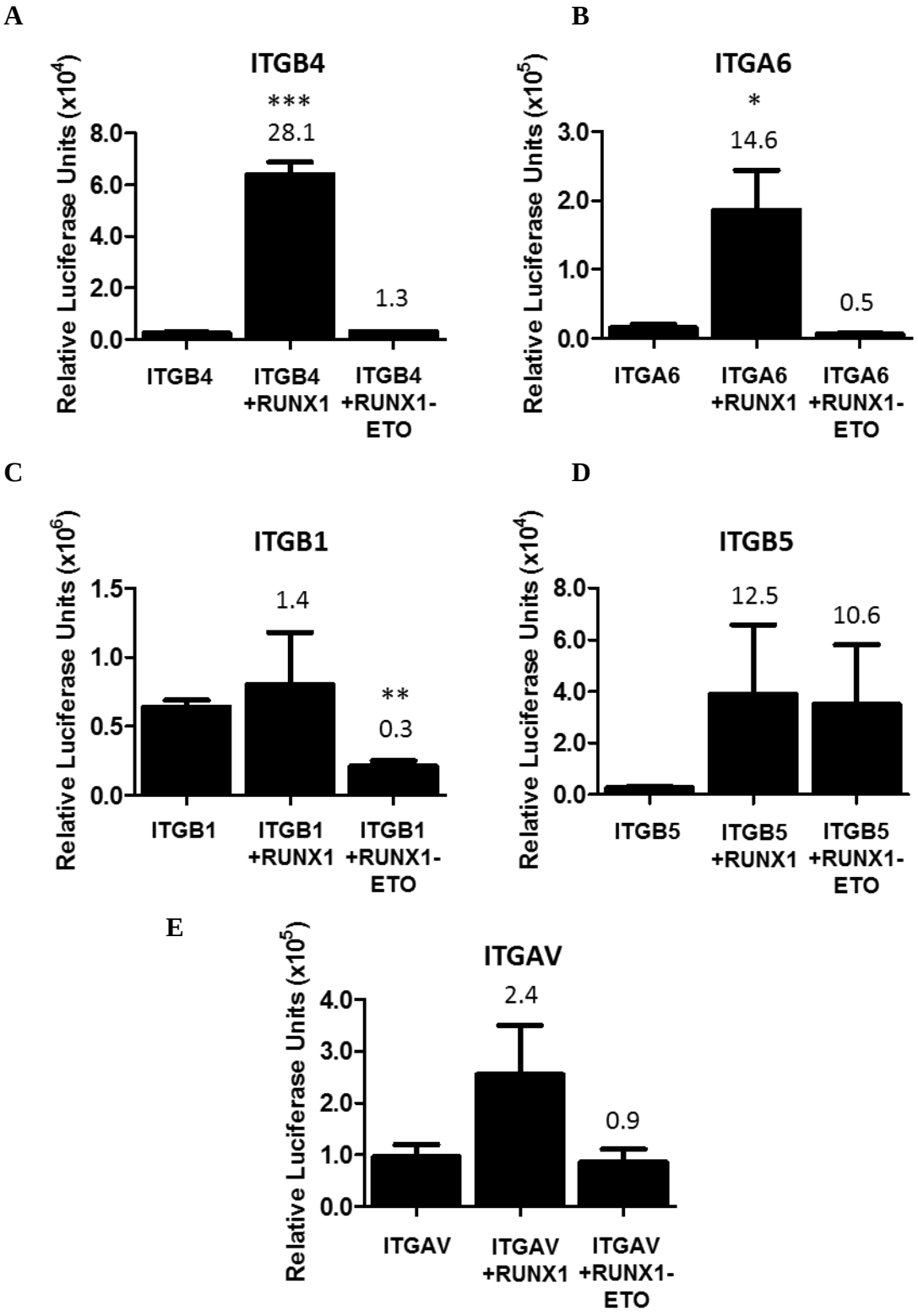


Figure 3.12 – RUNX1 and RUNX1-ETO effects on integrin promoters. K562 cells were transfected with an integrin promoter luciferase reporter construct (pXPG-ITGB4, pXPG-ITGA6, pXPG-ITGB1, pXPG-ITGB5 or pXPG-ITGAV) along with a construct expressing RUNX1 or RUNX1-ETO. After 24 hours, protein was extracted and luciferase activity was measured. Values are expressed as mean ±SEM (n=3, n=4 for ITGB1) and fold change in integrin promoter activity with RUNX1 or RUNX1-ETO overexpressed is shown. Statistical significance was determined using Students' *t* Test, ***p<0.001, **p<0.01, *p<0.05.

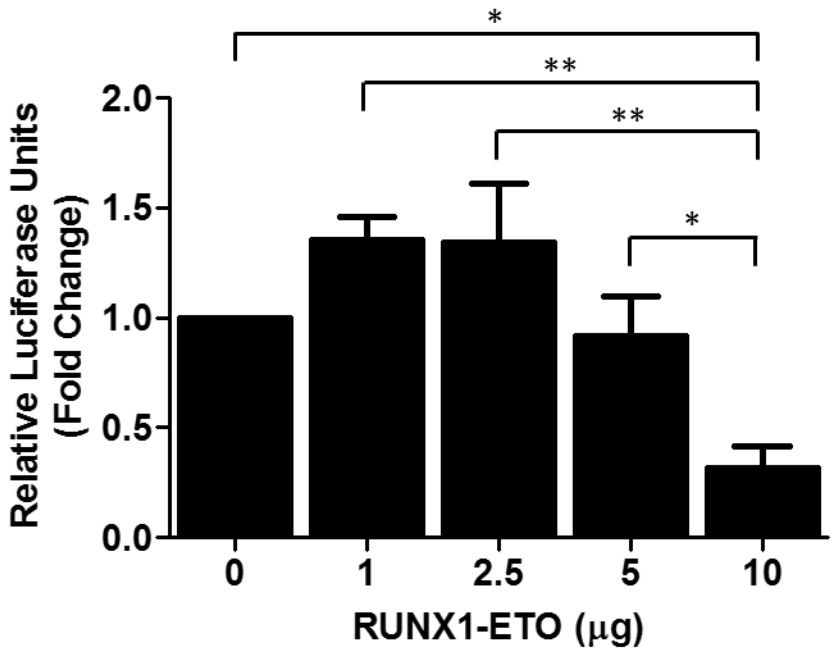


Figure 3.13 – Inhibition of RUNX1 activation of the ITGB4 promoter by RUNX1-ETO. K562 cells were transfected with pXPG-ITGB4 with 5 µg of RUNX1 expression plasmid and increasing amounts of RUNX1-ETO expression plasmid, as indicated. After 24 hours, protein was extracted and luciferase activity measured. Fold change relative to reporter activity in cells transfected with RUNX1 alone are depicted. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using one-way ANOVA, Newman Keuls Multiple Comparison Test, $^{**}p<0.01$, $^{*}p<0.05$.

3.3 Discussion

Several lines of evidence presented here suggest that expression of the α6β4 integrin receptor is regulated by RUNX1 in haematopoietic cells. Firstly, previously published microarray studies (Ichikawa *et al.* 2006, Valk *et al.* 2004), and a microarray study conducted by our research group (Oakford and Holloway, unpublished), showed altered *ITGB4* expression in haematopoietic cells with RUNX1 disrupted. These data suggest that *ITGB4* may be a target gene of RUNX1, although a limitation of microarray studies is that they do not distinguish between direct and indirect targets.

Secondly, analysis of publicly available ChIP-seq data (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Ptasinska *et al.* 2014, Tijssen *et al.* 2011, Trombly *et al.* 2015) demonstrated RUNX1 binding to the promoter of *ITGB4* in SKNO-1 cells, therefore confirming that *ITGB4* is a direct target of RUNX1 (Martens *et al.* 2012). Interestingly, although *ITGA6* was not identified as a potential RUNX1 target gene in the microarray studies, data from ChIP-seq studies suggest that it is also a direct target of RUNX1. RUNX1 was shown to bind to the ITGA6 promoter in megakaryocytes, SKNO-1 cells and Kasumi-1 cells, and RUNX1-ETO was detected at the ITGA6 promoter in Kasumi-1 cells (Martens *et al.* 2012, Ptasinska *et al.* 2014, Tijssen *et al.* 2011, Trombly *et al.* 2015). While ChIP-seq analysis provides information on DNA-protein interactions which occur in cells, a limitation of ChIP-seq is that it provides no information about the functional outcome of the transcription factor binding.

A third line of evidence indicating that RUNX1 regulates the ITGB4 and ITGA6 genes comes from reporter analysis of the respective promoters. RUNX1 was shown to have a functional effect on both ITGB4 and ITGA6 promoters. RUNX1 increased the activity of both of the promoters, while RUNX1-ETO decreased promoter activity, which is consistent with previous studies of other gene promoters describing RUNX1-ETO as a transcriptional repressor (Frank *et al.* 1995, Gelmetti *et al.* 1998, Wang *et al.* 1998). However, while RUNX1-ETO was shown to repress activity of the ITGB4 promoter in reporter assays, this is in contrast to available microarray data (Ichikawa *et al.* 2006, Valk *et al.* 2004) which show increased expression of *ITGB4* in cells positive for the t(8;21) chromosomal translocation compared to normal controls (Figure 3.14). In keeping with this, *ITGB4* was also found to be expressed at higher levels in the t(8;21) positive Kasumi-1 cells compared to KG-1a and K562 cells in the present study. These findings suggest

that RUNX1-ETO functions differently on the isolated ITGB4 promoter than in the context of the endogenous gene, and this warrants further investigation.

While RUNX1-ETO is commonly described as a transcriptional repressor, there is evidence that RUNX1-ETO can act as a transcriptional activator in reporter assays. RUNX1-ETO has been shown to work synergistically with RUNX1 to transactivate the M-CSF receptor promoter (Rhoades *et al.* 1996). Additionally, RUNX1-ETO can activate the transcription of the BCL-2 gene through the RUNX1 binding motif TGTGGT (Klampfer *et al.* 1996) and RUNX1-ETO can activate the G-CSF gene but independently of a RUNX1 binding site (Shimizu *et al.* 2000). Furthermore, in the present study, RUNX1-ETO was shown to increase activity of the ITGB5 promoter in reporter assays.

In recent studies, it has been shown that the truncated leukaemogenic form of RUNX1-ETO, RUNX1-ETO9a, which does not possess the C-terminal NcoR/SMRT-interacting domains, can act as a strong activator of transcription (Yan *et al.* 2004, Yan *et al.* 2006). This variant transcript can increase the expression of the integrin gene ITGB1 in murine bone marrow progenitor cells (Ponnusamy *et al.* 2014). In addition, down-regulation of RUNX1-ETO in Kasumi-1 cells results in decreased expression of *ITGB1*, suggesting that expression of *ITGB1* is dependent on the presence of the RUNX1-ETO fusion protein (Ponnusamy *et al.* 2014). These data are consistent with expression analysis in this study, which showed higher levels of ITGB1 mRNA in Kasumi-1 cells compared to K562 and KG-1a cells. Furthermore, a previous study has demonstrated that the RUNX1-ETO9a transcript is expressed in the Kasumi-1 cell line and in 27 of 37 individuals with t(8;21) AML (Yan *et al.* 2006). Taken together, higher levels of *ITGB4* in cells positive for the t(8;21) chromosomal translocation may be due to transcriptional activation by the variant transcript RUNX1-ETO9a. Further studies are therefore required to determine if RUNX1-ETO9a can activate the ITGB4 promoter.

Interestingly, PMA treatment of K562 cells increased the expression of ITGB4 and ITGA6 integrin genes, at least at the mRNA level. It has previously been shown that treatment of K562 cells results in stimulation of promoters such as TCRβ, MCSF and GM-CSF, which possess RUNX1 binding motifs (Zhang *et al.* 2004). While RUNX1 mRNA levels did not increase in response to PMA treatment, it has been reported that treatment with PMA results in increased transcriptional activity of the RUNX1 transcription factor in K562 cells due to phosphorylation, hence leading to increased

expression of RUNX1 target genes (Zhang *et al.* 2004). Therefore, the increase in *ITGB4* and *ITGA6* expression observed in K562 cells treated with PMA in this study could be due at least in part to increased transcriptional activity of RUNX1.

Although the ITGB5 gene was identified in the microarray studies (Ichikawa *et al.* 2006, Michaud *et al.* 2008, Wotton *et al.* 2008) to be a potential RUNX1 target gene, RUNX1 effects on the ITGB5 promoter were not statistically significant due to variability between the assays. Furthermore, in contrast to the other integrins, RUNX1-ETO was found to increase ITGB5 promoter activity. These data reflect the microarray studies where regulation of *ITGB5* by RUNX1 appears to be context dependent. Further investigation of *ITGB5* may therefore be useful in deciphering some of the less typical and context dependent functions of RUNX1 as a transcriptional regulator.

In conclusion, data presented here suggest that RUNX1 regulates the integrin genes ITGB4 and ITGA6, and thus expression of these genes may be altered in leukaemic cells with disrupted RUNX1. A question that remains is how RUNX1 regulates these genes and how this regulation is disrupted in leukaemia.

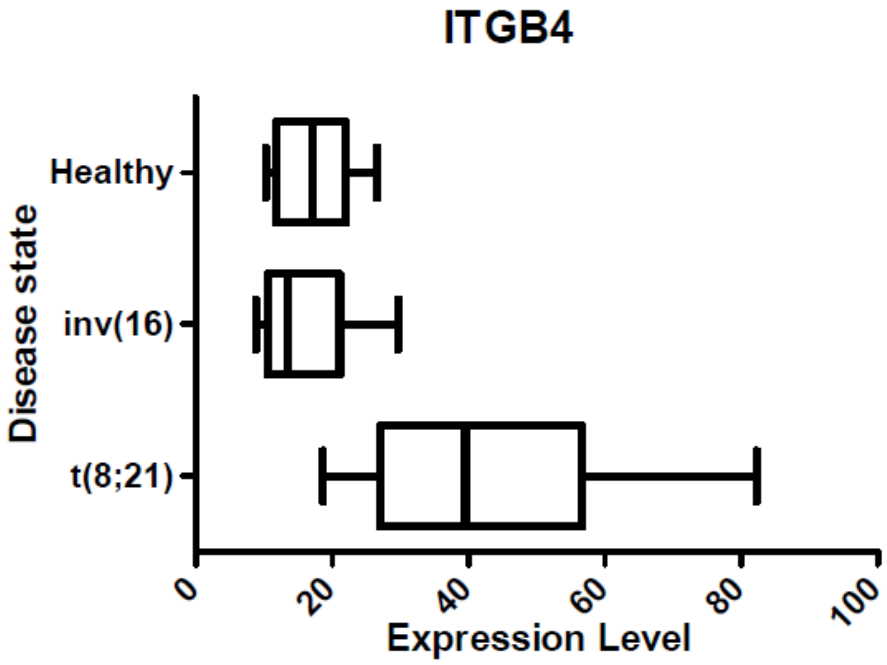


Figure 3.14 – Expression of *ITGB4* in leukaemic and healthy individuals. Expression levels of ITGB4 mRNA in primary leukaemic cells derived from individuals with leukaemia positive for the inv(16) or t(8;21) chromosomal rearrangements, based on published microarray data analysis (Valk *et al.* 2004) and extracted from the Leukemia Gene Atlas database (http://www.leukemia-gene-atlas.org/LGAtlas/).

Chapter 4

Regulation of *ITGB4* and *ITGA6* by RUNX1 in Myeloid Cells

4.1 Introduction

4.1.1 Complexity of RUNX1 Regulation of Gene Expression

The RUNX1 transcription factor controls expression of its target genes through a variety of different mechanisms. The structure of RUNX1 allows the transcription factor to exert either a positive or negative influence on the transcription of its target genes due to the presence of both transcriptional activation and inhibition domains at its C-terminus (Blyth *et al.* 2005). Furthermore, RUNX1 regulation of gene expression, as with many transcription factors, is complicated by the fact that it acts in complexes with other transcription factors to regulate its target genes (Giese *et al.* 1995, Gu *et al.* 2000, Huang *et al.* 2009, Mao *et al.* 1999, Zhang *et al.* 1996), its ability to direct epigenetic modification (Kitabayashi *et al.* 1998, Lutterbach *et al.* 2000, Oakford *et al.* 2010, Reed-Inderbitzin *et al.* 2006) and its ability to regulate genes through both promoters as well as distal regulatory regions such as enhancers (Bowers *et al.* 2010, Hernandez-Munain and Krangel 1994, Hernández-Munain and Krangel 2002, Meyers *et al.* 1993, Takahashi *et al.* 1995, Zhang *et al.* 1994).

Early studies described RUNX1 as a transcriptional activator, which bound to the consensus sequence TGT/cGGT in the promoters of its target genes (Meyers *et al.* 1993, Takahashi *et al.* 1995). The presence of a TGT/cGGT consensus site in regulatory regions was therefore used to identify potential target genes (Meyers *et al.* 1993, Takahashi *et al.* 1995, Zhang *et al.* 1994). However, subsequent research has demonstrated that RUNX1 is a relatively weak activator alone and therefore commonly regulates gene expression in a complex with other transcription factors.

Interaction of RUNX1 with other transcription factors was first reported in a study investigating regulation of the T-cell receptor β gene enhancer (Wotton *et al.* 1994). Both

RUNX1 and the ETS-1 transcription factor were found to bind to the enhancer, and ETS-1 and RUNX1 binding sites were identified directly adjacent to each other within the enhancer region (Wotton *et al.* 1994). ETS-1 and RUNX1 were found to cooperatively bind to the enhancer to form a high-affinity DNA-binding complex, but only when both binding sites were present (Wotton *et al.* 1994). In further studies, RUNX1 was also shown to cooperate with C/EBPα to synergistically activate the M-CSF receptor promoter (Zhang *et al.* 1996). RUNX1 physically interacted with C/EBPα in *in vitro* assays and simultaneous binding of the transcription factors to the DNA resulted in activation of the M-CSF receptor promoter by more than 60-fold (Zhang *et al.* 1996). This activation was dependent on the presence of both RUNX1 and C/EBP binding sites (Zhang *et al.* 1996). As well as ETS-1 and C/EBPα, RUNX1 can physically interact with other transcription factors important for haematopoiesis, such as GATA1 and FLI1, to activate gene promoters (Elagib *et al.* 2003, Huang *et al.* 2009, Waltzer *et al.* 2003).

In addition to influencing the transcriptional activity of its target genes, RUNX1 can affect the epigenetic status of genes by interacting with epigenetic modifiers, as reviewed recently (Brettingham-Moore *et al.* 2015). To activate gene expression, RUNX1 has been shown to interact with the histone acetyltransferases p300 and CBP (Kitabayashi *et al.* 1998). A study has shown that RUNX1 is required for the hyperacetylation of the GM-CSF promoter via the interaction with CBP (Oakford *et al.* 2010). Furthermore, RUNX1 can repress gene expression by interacting with HDACs, mSin3a and the histone methyltransferase SUV39H1 (Dannenberg *et al.* 2005, Durst and Hiebert 2004, Lutterbach *et al.* 1999, Reed-Inderbitzin *et al.* 2006) and a study has shown RUNX1 to repress and silence the CD4 gene by interacting with HDACs and SUV39H1 (Reed-Inderbitzin *et al.* 2006).

While much of the early work investigating RUNX1 function focussed on regulation of gene expression by promoter bound RUNX1, more recent genome-wide analysis has shown that RUNX1 binds more frequently to intragenic/intergenic regions compared to promoter regions (Beck *et al.* 2013, Wilson *et al.* 2010). These distal regulatory regions, such as enhancers, are often located several hundred kilobases either upstream or downstream of a gene promoter (Maston *et al.* 2006). For example, RUNX1 can regulate the GM-CSF gene through an upstream enhancer located 3 kb from the transcription start site (Bowers *et al.* 2010). In addition, RUNX1 is important for the assembly and function of the TCRδ enhancer, enabling c-Myb enhancer binding and activation of the gene

promoter (Hernández-Munain and Krangel 2002), and RUNX1 has been shown to function synergistically with c-Myb to activate the myeloperoxidase gene enhancer (Britos-Bray and Friedman 1997). Enhancers can function by interacting with the promoters of genes through DNA looping, which is often facilitated by transcription factor interactions and allows the enhancer and promoter to come into close proximity in the cell (Maston *et al.* 2006). RUNX1 has been shown to regulate the CD34 gene in this manner, through an enhancer located 18.8-19.6 kb downstream of the transcription start site (Levantini *et al.* 2011). The downstream enhancer region was found to physically interact with the CD34 promoter. Targeted mutagenesis of RUNX binding motifs within the enhancer led to disruption of this interaction and decreased CD34 expression in haematopoietic stem cells, suggesting that RUNX1 is required for these promoter-enhancer interactions (Levantini *et al.* 2011).

4.1.2 RUNX1 Regulation of ITGB4 and ITGA6 Integrin Genes

Regulation of gene expression is therefore complex with RUNX1 acting at multiple levels to regulate gene activity. Evidence presented in *Chapter 3* indicate that RUNX1 can bind to and regulate the ITGB4 and ITGA6 promoters, and therefore, the second aim of this study was to characterise the mechanisms through which RUNX1 regulates these genes.

4.2 Results

4.2.1 RUNX1 Regulation of the ITGA6 Promoter

4.2.1.1 Characterisation of the RUNX1 Responsive Region in the ITGA6 Promoter

Reporter assays described in *Chapter 3* indicate that RUNX1 activates the ITGA6 promoter in myeloid cells, as demonstrated by increased reporter activity when RUNX1 was overexpressed (Figure 3.12). Bioinformatic analysis also identified a number of potential RUNX1 binding motifs within the -675 bp to +242 bp region analysed in reporter assays (Table 3.1). To determine the region of the ITGA6 promoter through which RUNX1 is acting, a deletion construct (Del-1) was created to delete 6 of 9 putative RUNX1 binding motifs within the RUNX1 responsive region (Figure 4.1A). Of these 6 motifs, one is a 100% match to the RUNX1 consensus sequence (TGTGGT). The deletion construct included the region -140 bp to +242 bp relative to the transcription start site and removed this consensus motif at -237 to -232 bp (Figure 4.1A).

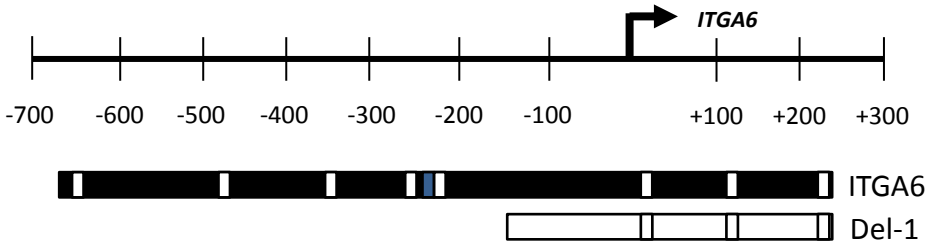
K562 cells were transfected with either the original ITGA6 promoter construct or the deletion construct along with the RUNX1 expression plasmid. Cells were harvested 24 hours after transfection and luciferase activity was measured. To determine the effect of RUNX1 on the ITGA6 promoter constructs, the fold change in promoter activity in transfected cells following RUNX1 overexpression was compared to cells transfected with the promoter construct alone. Overexpression of RUNX1 in K562 cells resulted in an increase in reporter activity of the ITGA6 promoter construct (Figure 4.1B). Deletion of the region from -675 bp to -140 bp significantly reduced RUNX1 activation of the promoter (Figure 4.1B; $p<0.001$). While RUNX1 responsiveness was not completely removed, these results suggest that a RUNX1 responsive region of the ITGA6 promoter is located -675 bp to -141 bp upstream of the transcription start site.

4.2.1.2 Effect of Mutation of the Consensus RUNX1 Binding Motif in the ITGA6 Promoter

To determine if RUNX1 was acting through the single consensus motif within the region -675 bp to -141 bp upstream of the transcription start site, it was mutated in the full length reporter construct creating an *EcoRV* recognition sequence (TGTGGT to GATATC) at -237 bp to -232 bp, following the protocol described in *Chapter 2, Section 2.2*. To confirm the *EcoRV* recognition sequence was created, the plasmid DNA was digested with *HindIII* and *EcoRV* restriction enzymes, with the expected size fragments of 6504 bp and 476 bp produced (Figure 4.2A). The plasmid DNA was also sequenced to confirm mutation of the consensus RUNX1 binding motif (Figure 4.2B).

K562 cells were then transfected with the wild type ITGA6, deletion and mutant reporter constructs (Figure 4.3A), along with the RUNX1 expression plasmid. Cells were harvested after 24 hours and luciferase activity was measured. Fold change in activity of each promoter construct with RUNX1 overexpressed was analysed. As described previously, RUNX1 activated the full-length reporter construct, but only had a minimal effect on the deletion construct (Figure 4.3B; Del-1). Mutation of the consensus RUNX1 binding motif resulted in a significant decrease in RUNX1 activation of the promoter, to a similar level observed with the deletion construct (Figure 4.3B; $p<0.01$). These data therefore suggest that RUNX1 activates the ITGA6 promoter through a classical RUNX1 binding motif located -237 bp to -232 bp upstream of the transcription start site.

A



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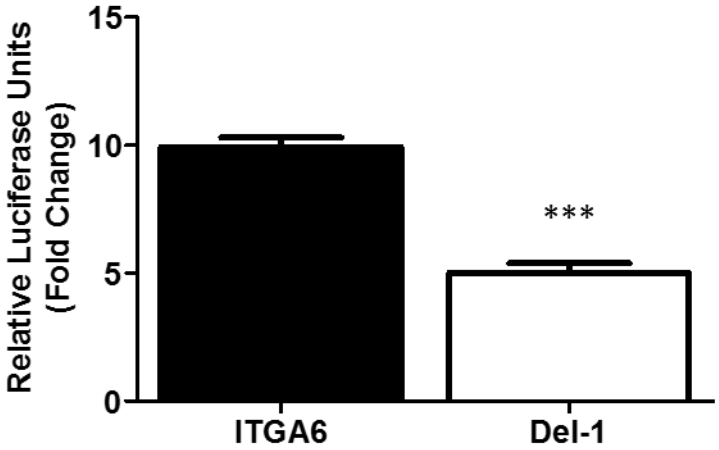
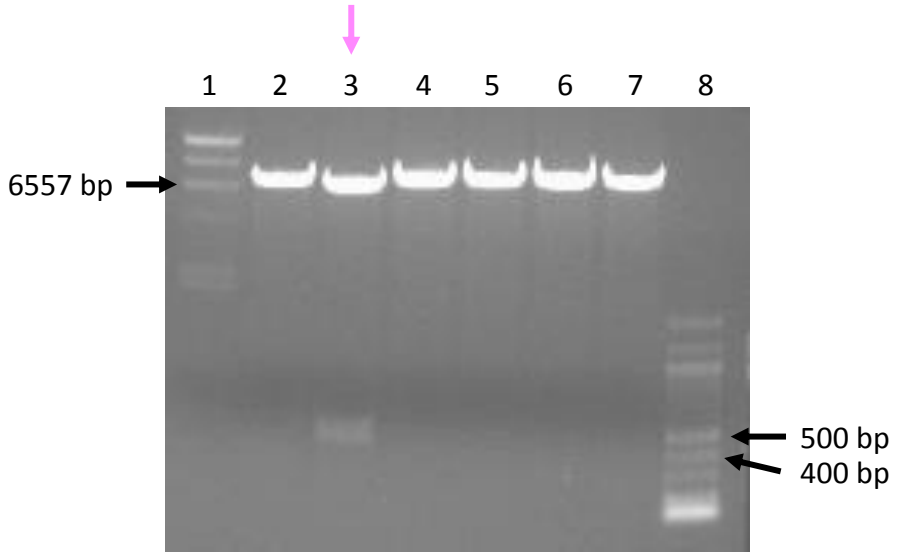


Figure 4.1 – Identification of a RUNX1 responsive region located -141 bp to -675 bp upstream of the transcription start site of *ITGA6*. A) Schematic representation of ITGA6 promoter regions analysed in reporter assays. ITGA6 promoter regions were cloned into the pXPG plasmid containing a luciferase gene. Scale indicates base pairs relative to the transcription start site (indicated by arrow). White boxes represent putative RUNX1 binding motifs and the blue box represents a motif with 100% match to the RUNX1 consensus sequence. **B)** K562 cells were transfected with the promoter reporter constructs either with or without the RUNX1 expression plasmid. Protein was isolated from transfected cells after 24 hours and analysed in a luciferase reporter assay. Fold change in activity of ITGA6 and Del-1 constructs following RUNX1 overexpression in K562 cells is shown. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using Student's *t* Test, ***$p<0.001$.

A



B

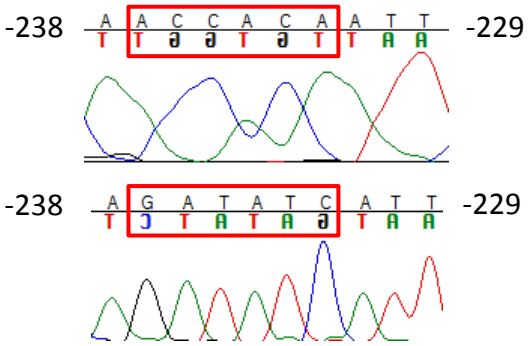
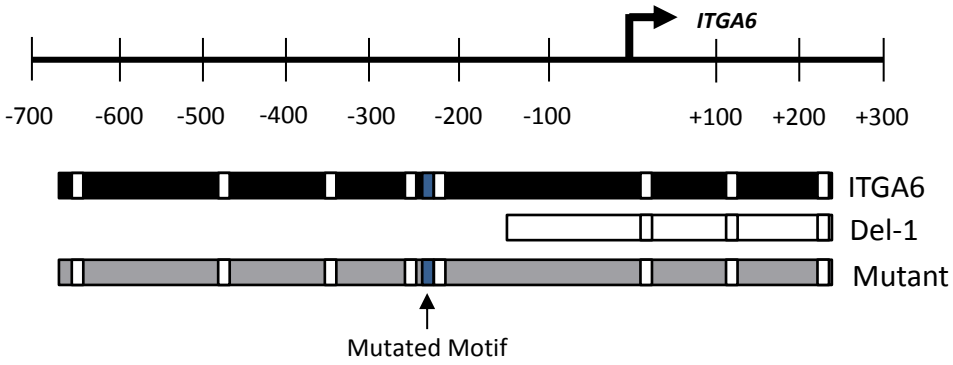


Figure 4.2 – Mutation of the consensus RUNX1 binding motif in the ITGA6 promoter. A) The RUNX1 consensus motif was mutated in the ITGA6 promoter creating an *EcoRV* recognition site, as described in *Chapter 2, Section 2.2*. The mutant promoter region was ligated into the pXPG plasmid and recombinant plasmids were transformed into *E.coli* cells. Single bacterial colonies were screened for the mutant promoter region by digestion with *HindIII* and *EcoRV* enzymes. Digested plasmid DNA was subjected to gel electrophoresis using a 1% agarose gel. Pink arrow shows the plasmid which is digested by *EcoRV*, suggesting incorporation of the mutant sequence. Lane 1: Lambda molecular weight marker; Lane 2-7: plasmid DNA from single bacterial colonies; Lane 8: 100 bp molecular weight marker. **B)** Screen shot of promoter regions, viewed in Sequencher 4.10.1. Red boxes show the normal and mutated RUNX1 consensus binding motif in ITGA6 and mutant promoter regions, respectively.

A



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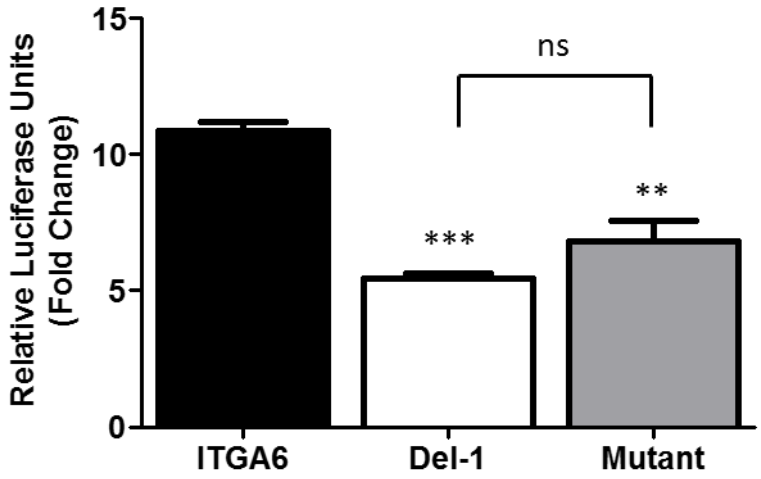


Figure 4.3 – RUNX1 regulates the ITGA6 promoter through a consensus RUNX1 binding motif. A) Schematic representation of ITGA6 promoter regions analysed in reporter assays. ITGA6 promoter regions were cloned into the pXPG plasmid containing a luciferase gene. Scale indicates base pairs relative to the transcription start site (indicated by arrow). White boxes represent putative RUNX1 binding motifs and the blue box represents a motif with 100% match to the RUNX1 consensus sequence. **B)** K562 cells were transfected with promoter reporter constructs either with or without the RUNX1 expression plasmid. Protein was isolated from transfected cells after 24 hours and analysed in a luciferase reporter assay. Fold change in activity of ITGA6, Del-1 and Mutant constructs following RUNX1 overexpression in K562 cells is shown. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using one-way ANOVA, Newman Keuls Multiple Comparison Test, ***p<0.001, **p<0.01, not significant (ns) p>0.05.

4.2.1.3 RUNX1 Binds to the Endogenous ITGA6 Promoter

Reporter assays indicate that RUNX1 can activate the ITGA6 promoter in myeloid cells, therefore binding of RUNX1 to the endogenous ITGA6 promoter was analysed using ChIP assays. RUNX1 binding to the ITGA6 promoter was analysed in KG-1a and Kasumi-1 cell lines. RUNX1 binding was not examined in K562 cells based on the less reliable data generated in genome-wide analysis in these cells, as described in *Chapter 3*, which may be a result of lower RUNX1 levels in these cells. KG-1a and Kasumi-1 cells were fixed with formaldehyde and DNA was sheared using sonication. DNA immunoprecipitated with a RUNX1 antibody was analysed by qPCR with primers that amplify a region adjacent to the RUNX1 consensus motif in the ITGA6 promoter. As a control for RUNX1 binding, the promoter of the Rhodopsin gene, which is only normally expressed in G-protein-coupled photoreceptors in the eye (Palczewski *et al.* 2000) and therefore expected to be silenced in haematopoietic cells, and additionally does not contain any RUNX consensus motifs, was also analysed for RUNX1 enrichment. In both KG-1a and Kasumi-1 cells, there was increased RUNX1 occupancy at the ITGA6 promoter compared to the Rhodopsin promoter (Figure 4.4). While enrichment at the ITGA6 promoter was statistically significant in KG-1a cells, statistical testing was not possible in Kasumi-1 cells as there was no PCR amplification of the Rhodopsin promoter in the RUNX1 ChIP in these cells.

Together, these data suggest that RUNX1 binds to and activates the ITGA6 promoter through a RUNX1 consensus motif.

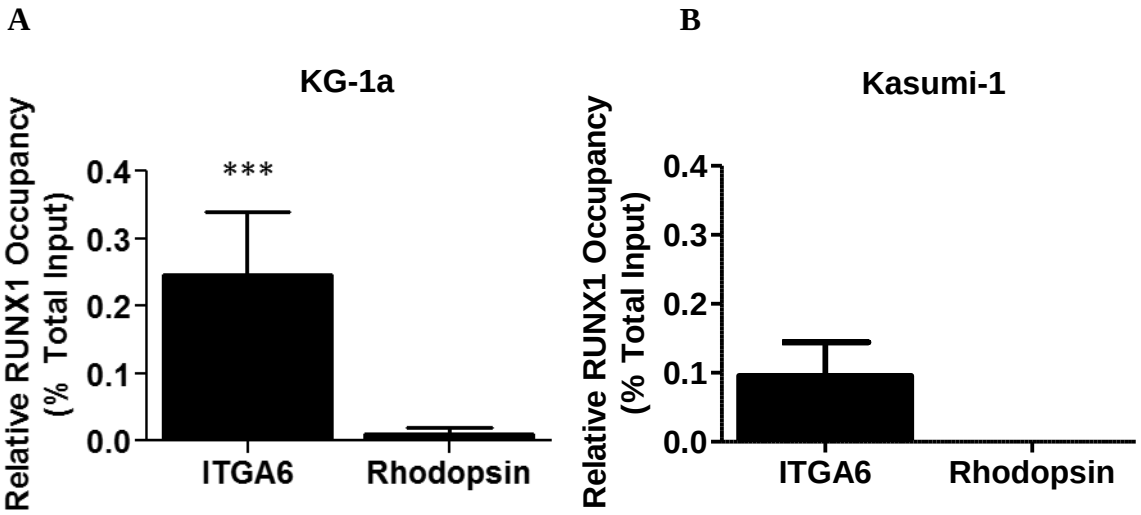


Figure 4.4 – RUNX1 binds the ITGA6 promoter in KG-1a and Kasumi-1 cells. A-B) ChIP assays were performed with an antibody against RUNX1 in **A)** KG-1a and **B)** Kasumi-1 cells. Immunoprecipitated DNA was analysed by qPCR with primers that amplify a region within the ITGA6 and Rhodopsin promoters. The data are shown as the ratio of immunoprecipitated DNA to total input DNA. Values are expressed as mean ±SEM (n=3-6). Statistical significance was determined using Student's *t* Test, ***$p<0.001$.

4.2.2 RUNX1 Regulation of the ITGB4 Promoter

4.2.2.1 Characterisation of the RUNX1 Responsive Region in the ITGB4 Promoter

Reporter assays described in *Chapter 3* indicate that RUNX1 activates the ITGB4 promoter in myeloid cells, as demonstrated by increased reporter activity when RUNX1 was overexpressed (Figure 3.12). To determine the region of the ITGB4 promoter through which RUNX1 is acting, deletion constructs were created to delete putative RUNX1 binding motifs in this region as described in *Chapter 3* (Figure 4.5A). Four deletion constructs were initially created to analyse regions -758 bp to +144 bp (Del-1), -295 bp to +144 bp (Del-2), -57 bp to +144 bp (Del-3) and -57 bp to +13 bp (Del-4) of the ITGB4 5'UTR (Figure 4.5A). K562 cells were transfected with either the original ITGB4 promoter construct or the deletion constructs (Del-1 to -4) along with the RUNX1 expression plasmid. Cells were harvested 24 hours after transfection and luciferase activity was measured. Fold change in activity of each promoter construct following RUNX1 overexpression was determined. To elucidate the effect of deletions to different regions of the ITGB4 promoter, all fold changes for the deletion constructs were determined relative to the fold change of the original ITGB4 promoter construct, which was set to 1 (Figure 4.5B). Deletion of the region from -1199 bp to -295 bp of the ITGB4 gene had no significant effect on RUNX1 activation of the ITGB4 promoter, as RUNX1 activated the Del-1 and Del-2 reporter constructs similarly to the original (-1199 bp to +144 bp) ITGB4 reporter construct (Figure 4.5B). However, further deletion of the promoter from -295 bp to -58 bp, to generate a -57 bp to +144 bp construct (Del-3), resulted in a significant decrease in RUNX1 activation of the ITGB4 promoter (Figure 4.5B; $p<0.01$). Further deletion to generate a minimal -57 bp to +13 bp promoter construct (Del-4), resulted in a further decrease in RUNX1 activation, although this was not statistically different to Del-3 (Figure 4.5B; $p>0.05$).

These data suggest that the region from -295 bp to -58 bp upstream of the transcription start site contains a RUNX1 responsive region. The further decrease in reporter activity observed when removing the region from +13 bp to +144 bp is likely due to an effect on general transcription machinery assembly. To confirm these data, the -295 bp to -58 bp region was deleted from the larger Del-1 (-758 bp to +144 bp) construct (Figure 4.5A). Specific deletion of this region (Del-5) resulted in a decrease in RUNX1 activation of the ITGB4 promoter to a level similar to that seen when the entire region from -758 bp to -

58 bp was deleted (Figure 4.5C), confirming that this region is required for activation of the ITGB4 promoter by RUNX1.

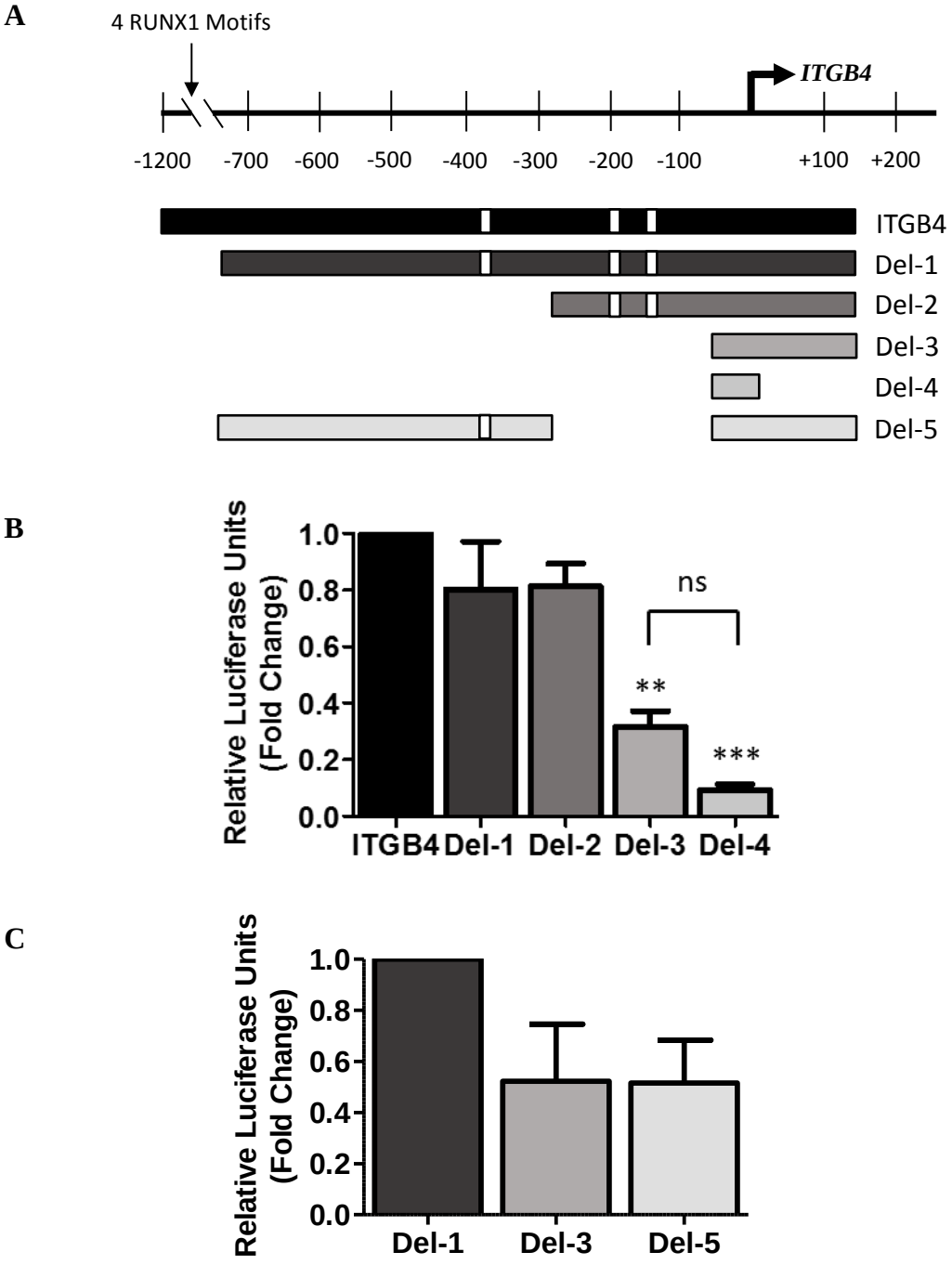


Figure 4.5 – Identification of a RUNX1 responsive region within the ITGB4 promoter located -295 to -58 bp upstream of the transcription start site. A) Schematic representation of ITGB4 promoter regions analysed in reporter assays. ITGB4 promoter regions were cloned into the pXPG plasmid containing a luciferase gene for reporter assays. Scale indicates base pairs relative to the transcription start site (indicated by arrow). White boxes represent putative RUNX1 binding motifs (with a further 4 within the -700 to -1200 region of ITGB4 not depicted), although none are 100% matches to the consensus sequence. **B-C)** K562 cells were transfected with promoter reporter constructs either with or without the RUNX1 expression plasmid. Protein was isolated from transfected cells after 24 hours and analysed in a luciferase reporter assay. Relative fold change in activity of constructs following RUNX1 overexpression in K562 cells is shown. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using one-way ANOVA, Newman Keuls Multiple Comparison Test, ***p<0.001, **p<0.01, not significant (ns) p>0.05.

4.2.2.2 Mutation of a Potential RUNX1 Binding Motif in the ITGB4 Promoter

Within the RUNX1 responsive region identified in the ITGB4 promoter, located -295 bp to -58 bp upstream of the transcription start site (Section 4.2.2.1), there are two putative RUNX1 binding motifs, GGTGGC at -196 bp to -201 bp and GCCGCA at -135 bp to -140 bp. Therefore, to determine which of these motifs may be required for RUNX1 activation of the promoter, another deletion construct (Del-6) was created to delete only one of the putative RUNX1 binding sites (Figure 4.6A). Deletion from -295 bp to -176 bp of the transcription start site and thus removing the motif at -196 bp to -201 bp unexpectedly resulted in a significant increase in RUNX1 activation of the promoter (Figure 4.6B, $p<0.01$). These results therefore suggest that this site is not responsible for RUNX1 activation of the promoter and that this activity is located within -58 bp to -175 bp of the ITGB4 gene.

A single putative RUNX1 binding motif at -135 bp to -140 bp is present in the region from -58 bp to -175 bp shown to be responsive to RUNX1. Therefore, to determine if RUNX1 is acting through this putative RUNX1 binding motif (GCCGCA), the motif was mutated within the reporter construct containing the region -295 bp to +144 bp (Del-2), creating a *BglII* recognition sequence (GCCCGA to AGATCT), following the protocol described in *Chapter 2, Section 2.2*. To confirm the *BglII* recognition sequence was created, the plasmid DNA was digested with *BglII* restriction enzyme, with the expected sized fragments of 6343 bp and 174 bp produced (Figure 4.7A). The plasmid DNA was also sequenced to confirm mutation of the potential RUNX1 binding motif (Figure 4.7B)

K562 cells were transfected with Del-2 (-295 bp to +144 bp), Del-3 (-57 bp to +144 bp) and Mutant constructs (Figure 4.6A) along with the RUNX1 expression plasmid. Cells were harvested after 24 hours and luciferase activity was measured. Fold change in activity of each promoter construct with RUNX1 overexpressed was analysed. As described previously, RUNX1 activated the Del-2 construct, but only had a minimal effect on the Del-3 construct (Figure 4.6C). Mutation of the RUNX1 binding motif had no effect on RUNX1 activation of the promoter, therefore suggesting that RUNX1 does not activate the ITGB4 promoter specifically through this sequence (Figure 4.8C).

Put together, these data demonstrate that while the region upstream of the ITGB4 transcription start site contains a number of potential RUNX1 binding motifs, none of

these contributes to RUNX1 activation of the promoter in reporter assays. However, a RUNX1 responsive region is located at -58 bp to -175 bp, suggesting that RUNX1 is recruited to this region through an alternate mechanism.

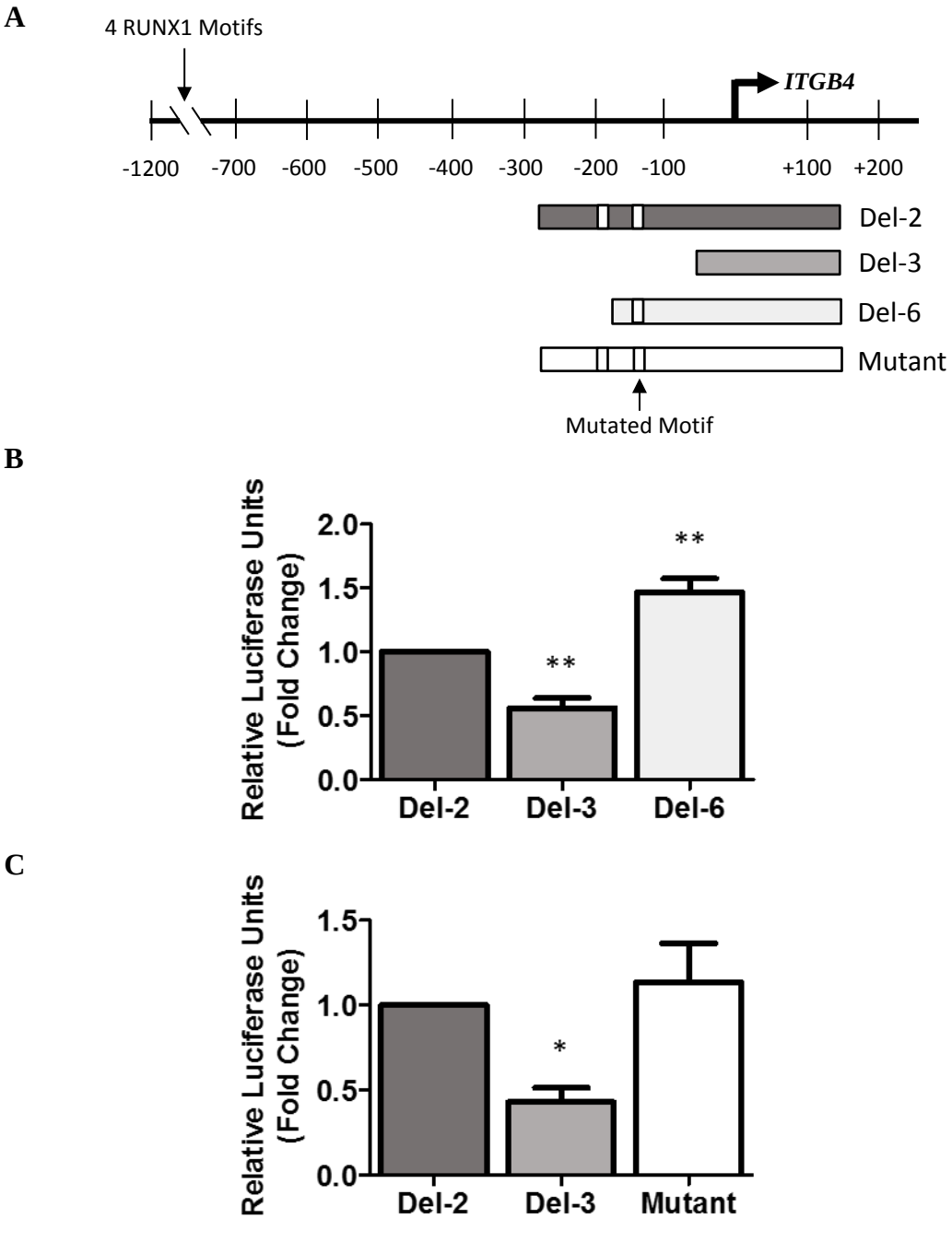


Figure 4.6 – RUNX1 does not activate the ITGB4 promoter through a RUNX1 binding motif. A) Schematic representation of ITGB4 promoter regions analysed in reporter assays. ITGB4 promoter regions were cloned into the pXPG plasmid containing a luciferase gene for reporter assays. Scale indicates base pairs relative to the transcription start site (indicated by arrow). White boxes represent putative RUNX1 binding motifs (with a further 4 within the -700 to -1200 region of ITGB4 not depicted), although none are 100% matches to the consensus sequence. **B-C)** K562 cells were transfected with promoter reporter constructs either with or without the RUNX1 expression plasmid. Protein was isolated from transfected cells after 24 hours and analysed in a luciferase reporter assay. Relative fold change in activity of constructs following RUNX1 overexpression in K562 cells is shown. Values are expressed as mean ±SEM (n=6). Statistical significance was determined using one-way ANOVA, Newman Keuls Multiple Comparison Test, $^{**}p<0.01$, $^{*}p<0.05$.

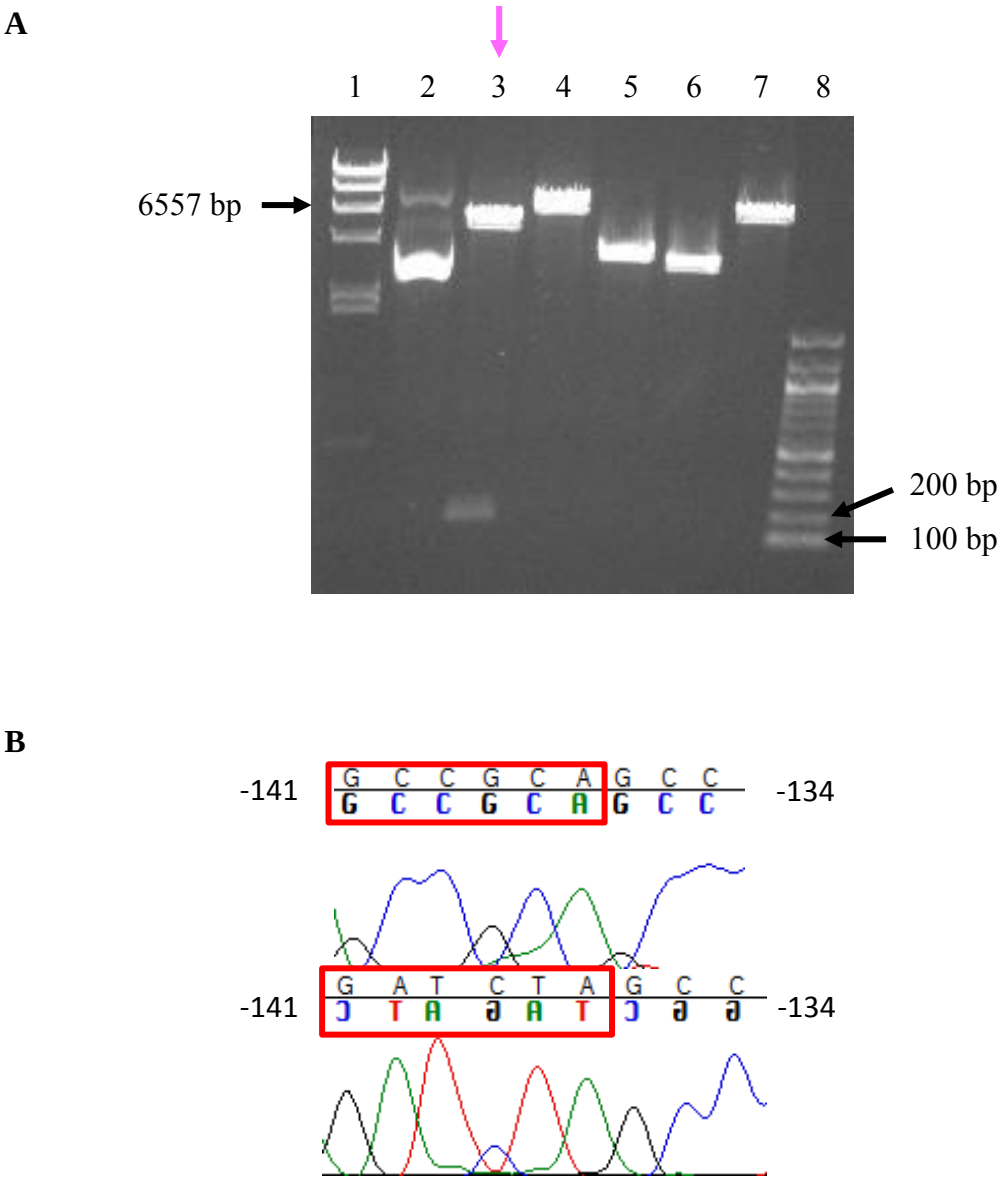


Figure 4.7 – Mutation of a potential RUNX1 binding motif in the ITGB4 promoter. **A)** A RUNX1 binding motif was mutated in the ITGB4 promoter creating a *BglII* recognition site, as described in *Chapter 2, Section 2.2*. The mutant promoter region was ligated into the pXPG plasmid and recombinant plasmids were transformed into *E.coli* cells. Single bacterial colonies were screened for the mutant promoter region by digestion with *BglII* enzyme. Digested plasmid DNA was subjected to gel electrophoresis using a 1% agarose gel. Pink arrow shows the plasmid which is digested by *BglII*, suggesting incorporation of the mutant sequence. Lane 1: Lambda molecular weight marker; Lane 2-7: plasmid DNA from single bacterial colonies; Lane 8: 100 bp molecular weight marker. **B)** Screen shot of promoter regions, viewed in Sequencher 4.10.1. Red boxes show the normal and mutated RUNX1 binding motif in ITGB4 and mutant promoter regions, respectively.

4.2.2.3 RUNX1 Binds to the Endogenous ITGB4 Promoter

Reporter assays indicate that RUNX1 can activate the ITGB4 promoter in myeloid cells. Therefore, binding of RUNX1 to the endogenous ITGB4 promoter was analysed using ChIP assays. RUNX1 binding to the ITGB4 promoter was analysed in KG-1a and Kasumi-1 cell lines. As described previously, KG-1a and Kasumi-1 cells were fixed with formaldehyde and DNA was sheared using sonication. DNA immunoprecipitated with a RUNX1 antibody was analysed by qPCR with primers that amplify a region adjacent to the RUNX1 responsive region in the ITGB4 promoter. Again, as a control for RUNX1 binding, the promoter of the Rhodopsin gene was also analysed for RUNX1 enrichment. In both KG-1a and Kasumi-1 cells, there was increased RUNX1 occupancy at the ITGB4 promoter compared to the Rhodopsin promoter (Figure 4.8)

Together, these data suggest that RUNX1 binds to and activates the ITGB4 promoter, however, unlike *ITGA6*, RUNX1 does not bind to a consensus site within the promoter and therefore recruitment of RUNX1 to the promoter must occur through an alternate mechanism.

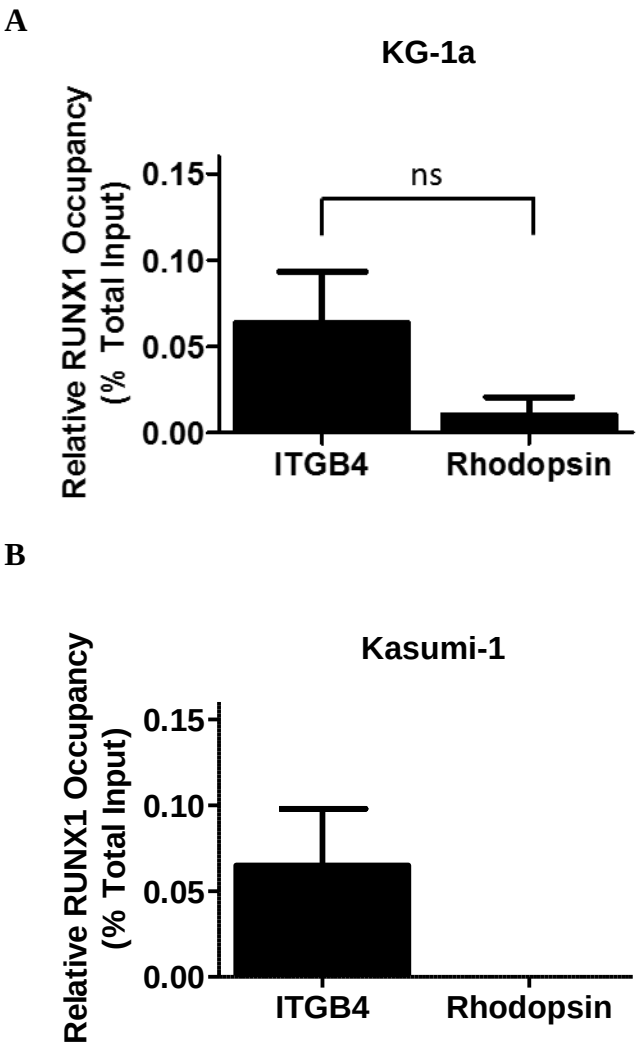


Figure 4.8 – RUNX1 binds the ITGB4 promoter in KG-1a and Kasumi-1 cells. A-B) ChIP assays were performed with an antibody against RUNX1 in **A)** KG-1a and **B)** Kasumi-1 cells. Immunoprecipitated DNA was analysed by qPCR with primers that amplify a region within the ITGB4 and Rhodopsin promoters. The data are shown as the ratio of immunoprecipitated DNA to total input DNA. Values are expressed as mean ±SEM (n=3-6). Statistical significance was determined using Student's *t* Test, ns $p>0.05$. In B), binding was not detected at the Rhodopsin region.

4.2.3 Regulation of ITGB4 by Distal Regions

4.2.3.1 Identification of an ITGB4 Enhancer

Regulation of the ITGB4 gene is altered in cells in which RUNX1 is disrupted (Ichikawa *et al.* 2006, Valk *et al.* 2004) and RUNX1 binds to the promoter in myeloid cells. However, the failure to find a RUNX1 binding motif through which it functions at the ITGB4 promoter suggests that its regulation of *ITGB4* is complex. Higher order chromatin structures and complex interactions, which are not assessed in reporter assays, may therefore also contribute to RUNX1 regulation of the promoter. Hence, to determine if the ITGB4 promoter interacts with other regions of the genome, Chromatin Interaction Analysis with Paired-End Tag Sequencing (ChIA-PET) data (Fullwood *et al.* 2009) from the ENCODE project (ENCODE 2012) was interrogated. The specific data set analysed was produced in K562 cells and identifies DNA loops between regions of the genome that coincide with RNA polymerase II binding (GSM970213).

Analysis of the ChIA-PET data revealed three interactions involving the ITGB4 promoter; a shorter range interaction with a region located -1,805 bp to -3,459 bp upstream of the transcription start site; a longer range interaction with a region located +8491 bp to +10,343 bp downstream; and another longer range interaction with a region located -12,053 bp to -14,003 bp upstream (Figure 4.9). While these interactions are relatively weak (the strength indicated by grey scale), this may reflect the low levels of *ITGB4* expression in K562 cells (Figure 3.6A). To determine whether any of the regions interacting with the ITGB4 promoter represent putative enhancer elements, the histone modification data from the ENCODE project (ENCODE 2012) was analysed for H3K4me1 and H3K27ac histone modifications representative of enhancer regions (Heintzman *et al.* 2009, Rada-Iglesias *et al.* 2011). Only one of these regions, located -12,053 bp to -14,003 bp upstream of the *ITGB4* transcription start site, situated towards the end of a SAP30BP gene, displayed chromatin features suggestive of an enhancer element. Both H3K4me1 and H3K27ac peaks were present, as well as a DNase hypersensitive site (Figure 4.9). Additionally, the H3K4me3 mark which is characteristic of promoters (Heintzman *et al.* 2007) was absent from this region, therefore suggesting that the region is not a gene promoter.

In contrast, similar analysis of the ITGA6 promoter failed to identify a putative enhancer element. While two short-range, localised interactions; one located +3,330 bp to +7270

downstream and another located -3240 bp to -4764 bp upstream of the *ITGA6* transcription start site were detected (Figure 4.10), unlike *ITGB4*, neither of these regions displayed chromatin features suggestive of enhancer regions, with no enrichment for H3K27ac and H3K4me1, and no DNase hypersensitivity detected (Figure 4.10).

To determine if RUNX1 may bind to the potential enhancer region for the ITGB4 promoter, located -12,053 bp to -14,003 bp upstream of *ITGB4*, ChIP-seq data as described in *Chapter 3* were interrogated for RUNX1 binding at this region (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Ptasinska *et al.* 2014, Tijssen *et al.* 2011, Trombly *et al.* 2015). RUNX1 was found to bind to the potential ITGB4 enhancer region in CD34+ and Kasumi-1 cells (Figure 4.11). Together, these data suggest that RUNX1 can be recruited to this upstream region, which interacts with the ITGB4 promoter and is associated with chromatin features consistent with an enhancer element.

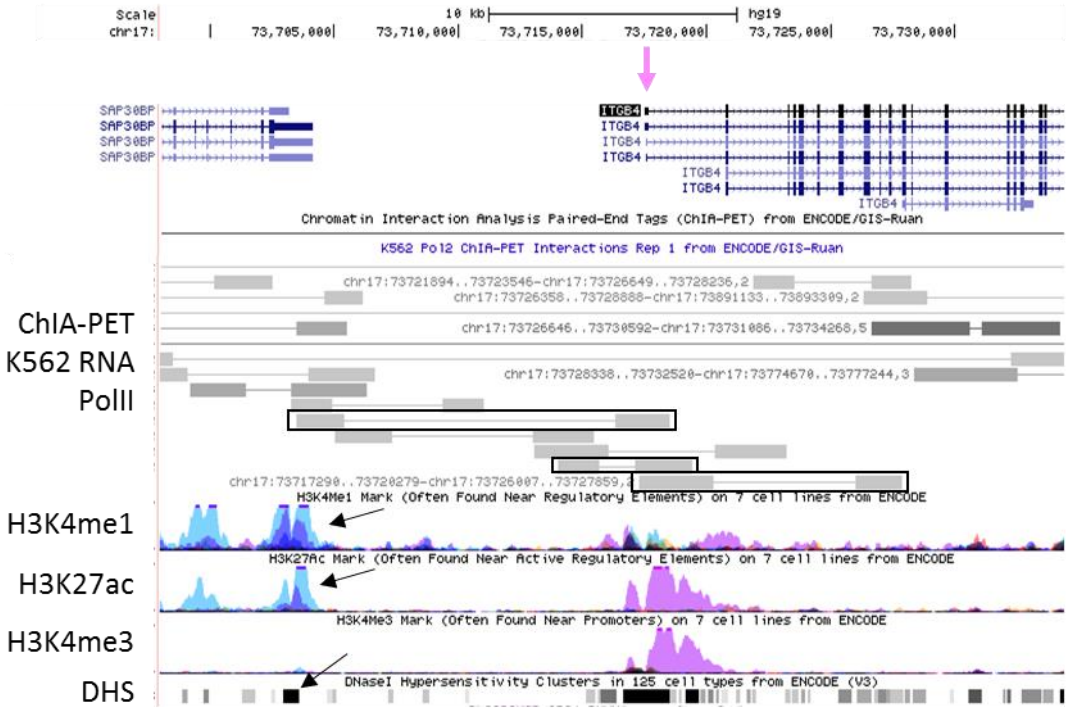


Figure 4.9 – Identification of a potential enhancer region upstream of the ITGB4 promoter. A screen shot of ChIA-PET data, histone modification ChIP-seq data and DNase hypersensitive site (DHS) data from the ENCODE project (ENCODE 2012) visualised in the UCSC Genome Browser (Kent *et al.* 2002) is shown. ChIA-PET data were analysed for regions of the genome which interact with the ITGB4 promoter. The ITGB4 promoter is shown by the pink arrow. Three regions of DNA found to interact with the ITGB4 promoter through RNA polymerase II in K562 cells were identified. The DNA loops of the three interacting regions of DNA are shown in the black boxes. Grey-scale of DNA loops indicates the strength of the interaction (darker shade is a stronger interaction whereas lighter shade is a weaker interaction). Histone modification ChIP-seq data were analysed for histone marks characteristic of enhancer and promoter regions (characteristic peaks are shown by black arrows). DNase hypersensitive site data were also analysed and hypersensitive sites are shown by the black arrow. ITGB4 and SAP30BP genes are represented at the top of the figure with exons shown as bars. The major transcript of *ITGB4* is in black, while the major transcript of *SAP30BP* is in dark blue.

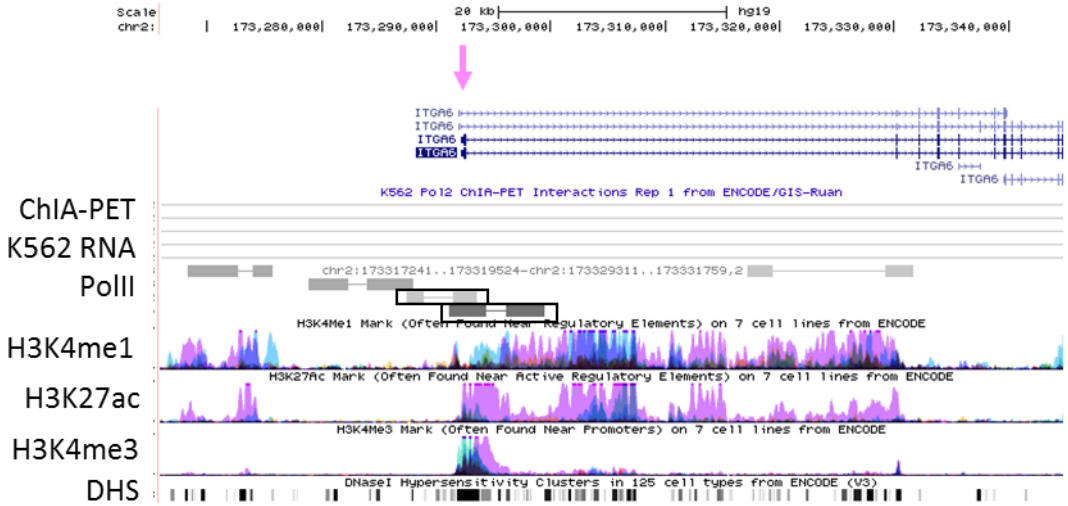


Figure 4.10 – Analysis of DNA loops involving the ITGA6 promoter. A screen shot of ChIA-PET data, histone modification ChIP-seq data and DNase hypersensitive site (DHS) data from the ENCODE project (ENCODE 2012) visualised in the UCSC Genome Browser (Kent *et al.* 2002) is shown. ChIA-PET data were analysed for regions of the genome which interact with the ITGA6 promoter. The ITGA6 promoter is shown by the pink arrow. Two regions of DNA found to interact with the ITGA6 promoter through RNA polymerase II in K562 cells were identified. The DNA loops of the two interacting regions of DNA are shown in the black boxes. Grey-scale of DNA loops indicates the strength of the interaction (darker shade is a stronger interaction whereas lighter shade is a weaker interaction). Histone modification ChIP-seq data were analysed for histone marks characteristic of enhancer and promoter regions. DNase hypersensitive site data were also analysed for accessible DNA. The ITGA6 gene is represented at the top of the figure with exons shown as bars. The major transcript is highlighted in dark blue.

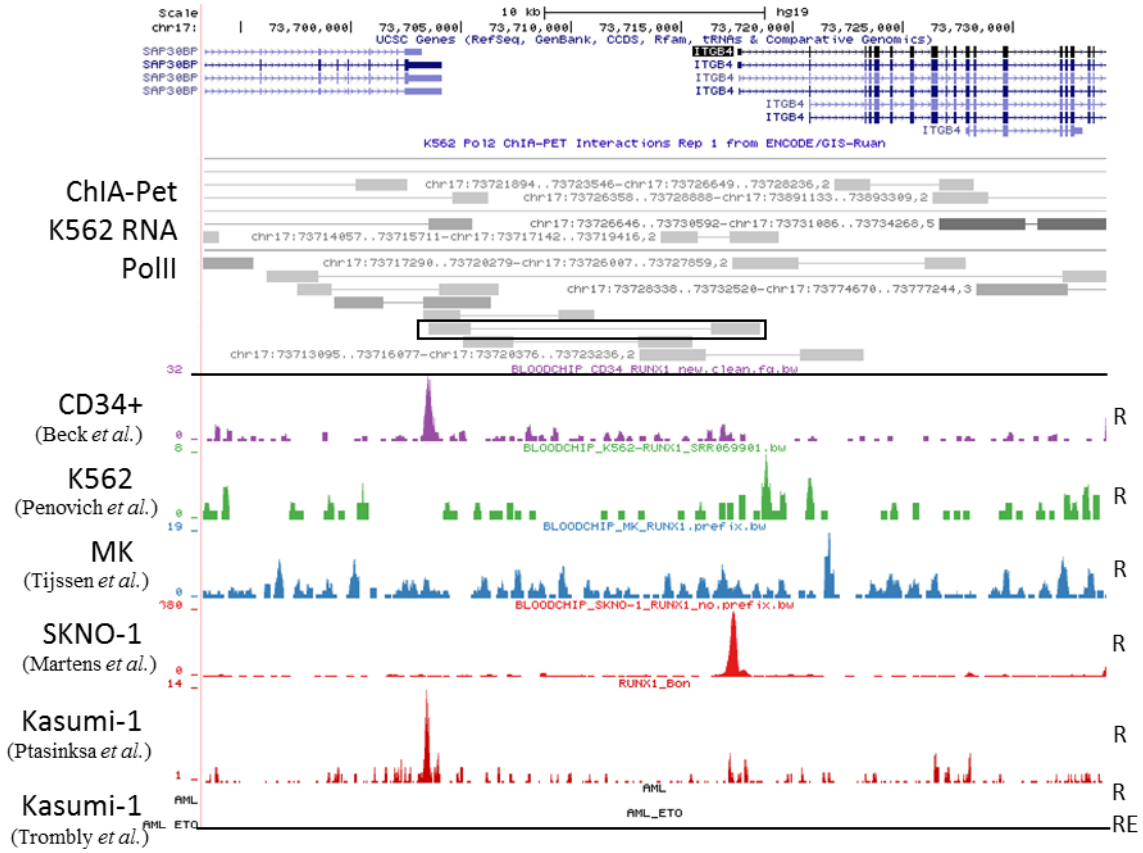


Figure 4.11 – RUNX1 binding at the potential ITGB4 enhancer in ChIP-seq studies. A screen shot of ChIA-PET data from the ENCODE project (ENCODE 2012) and data from ChIP-seq studies (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Ptasinska *et al.* 2014, Tijssen *et al.* 2011, Trombly *et al.* 2015) visualised in UCSC Genome Browser (Kent *et al.* 2002) is shown. The DNA loop involving the potential ITGB4 enhancer region and ITGB4 promoter is outlined in a black box. The left grey rectangle represents the potential enhancer region and the right grey rectangle represents the ITGB4 promoter region. RUNX1 (R) and RUNX1-ETO (RE) ChIP-seq studies are shown for different cell types. Peaks and lines represent RUNX1/RUNX1-ETO binding. ITGB4 and SAP30BP genes are represented at the top of the figure with exons shown as bars. The major transcript of *ITGB4* is in black, while the major transcript of *SAP30BP* is in dark blue.

4.2.3.2 Characterisation of RUNX1 Effects on a Potential ITGB4 Enhancer

In combination, the ChIA-PET, histone modification ChIP-seq and DHS data when mapped to the human genome suggest the presence of an enhancer, although at the 5' end of the ~2 kb region identified as interacting with the ITGB4 promoter by ChIA-PET (Figure 4.12). Further, this 5' region also displays RUNX1 binding in CD34+ and Kasumi-1 cells (Beck *et al.* 2013, Ptasinska *et al.* 2014) (Figure 4.12). However, on further analysis of the potential enhancer sequence using the MatInspector tool in the Genomatix bioinformatics suite (http://www.genomatix.de/), a consensus RUNX1 binding motif (TGTGGT) was identified, but towards the 3' end of the region (Figure 4.12). To localise RUNX1 binding to the putative enhancer, the 5' and 3' ends of the interacting region were analysed for RUNX1 binding in ChIP assays and for RUNX1 activity in reporter assays.

RUNX1 binding to the 5' and 3' ends of the potential ITGB4 enhancer region was analysed in KG-1a cells. As described previously, KG-1a cells were fixed with formaldehyde and DNA was sheared using sonication. DNA immunoprecipitated with a RUNX1 antibody was analysed by qPCR with primers, which amplify the 5' and 3' regions of the potential enhancer (Figure 4.13A). Again, as a control for RUNX1 binding, the promoter of the Rhodopsin gene was also analysed for RUNX1 enrichment. In KG-1a cells, there was increased enrichment of RUNX1 at the ITGB4 promoter as observed previously, and RUNX1 was also enriched at the 5' end of the potential enhancer (Distal-1), compared to the Rhodopsin promoter (Figure 4.13B). This is consistent with the available ChIP-seq data (Beck *et al.* 2013, Ptasinska *et al.* 2014) (Figure 4.11). Interestingly, there was no RUNX1 occupancy detected at the 3' end of the potential enhancer (Distal-2) containing the consensus RUNX1 binding motif (Figure 4.13B).

Since RUNX1 was shown to bind to the Distal-1 region, RUNX1 binding at this region was also analysed in Kasumi-1 cells. In contrast to KG-1a cells, no RUNX1 binding was detected at the Distal-1 region in Kasumi-1 cells, while RUNX1 was significantly enriched at the ITGB4 promoter as expected (Figure 4.13C). Together, these data suggest that RUNX1 may bind to the Distal-1 region in a cell-type specific manner. The data also suggest that although the Distal-2 region contains a consensus RUNX1 binding motif, the site is not functional, at least in the cell types examined here.

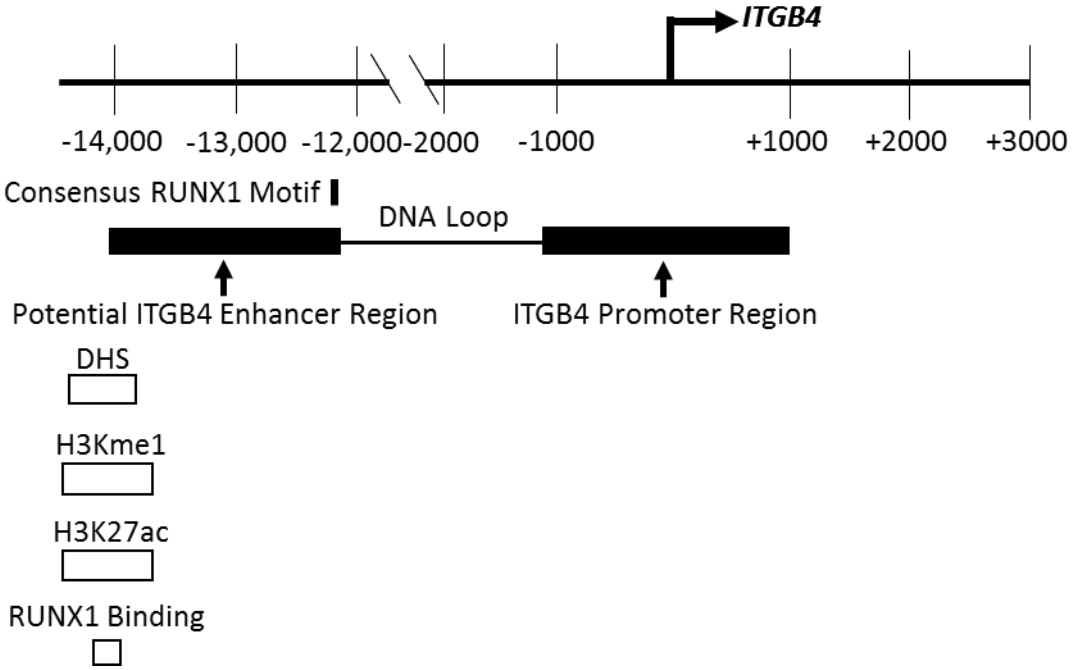


Figure 4.12 – Potential ITGB4 enhancer region mapped to the human genome. Schematic representation of potential regulatory regions of the ITGB4 gene. The DNA loop identified in ChIA-PET data from the ENCODE project (ENCODE 2012) involving the potential ITGB4 enhancer region and ITGB4 promoter is shown. A DNase hypersensitive site (DHS) (ENCODE 2012), histone marks H3K4me1 and H3K27ac (ENCODE 2012), and RUNX1 binding in CD34+ (Beck *et al.* 2013) and Kasumi-1 cells (Ptasinska *et al.* 2014) were detected at the 5' end of the potential enhancer region. A consensus RUNX1 binding motif was detected at the 3' end of the potential enhancer region. Scale indicates bases pairs relative to the transcription start site (indicated by arrow).

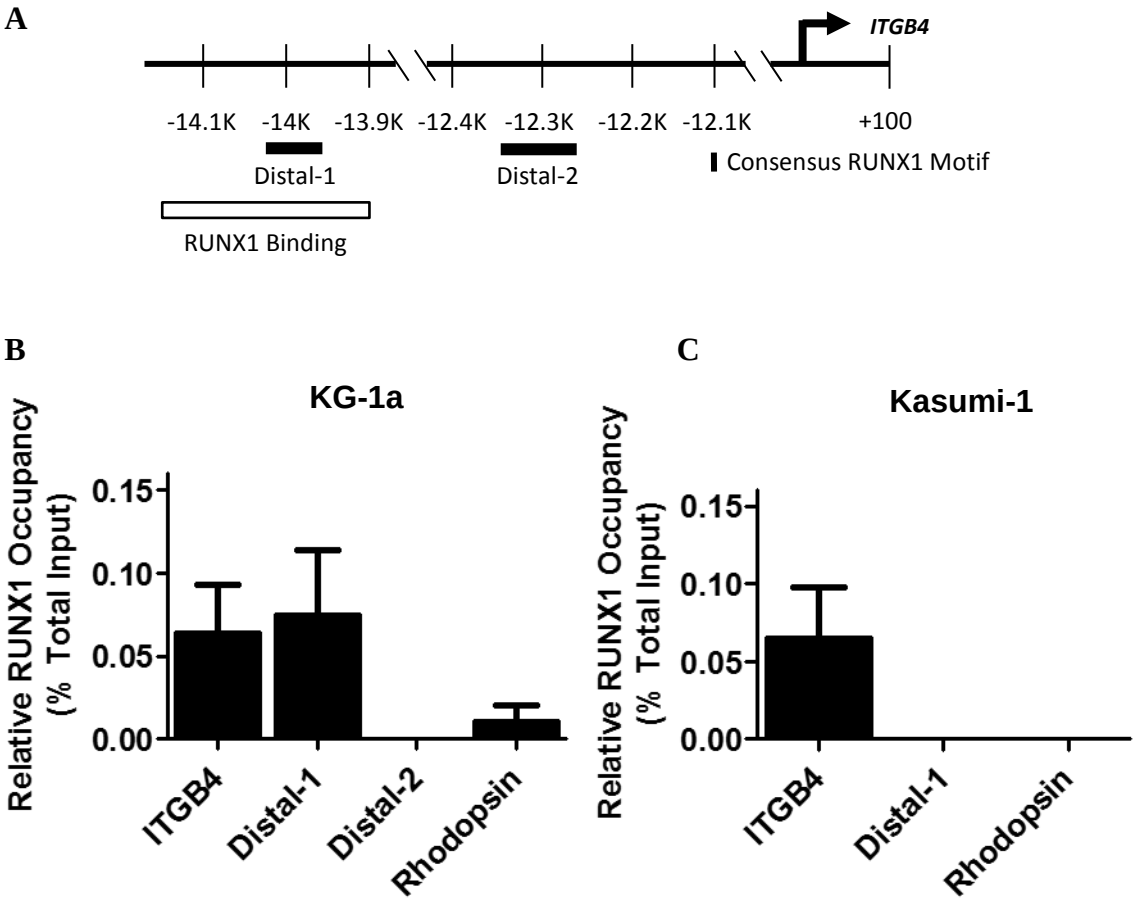


Figure 4.13 – RUNX1 binding at the potential ITGB4 enhancer region. A) Primers were designed for ChIP assay to amplify the region -13,957 bp to -14,020 bp (Distal-1) and the region -12,246 bp to -12,354 bp (Distal-2) of the ITGB4 gene in qPCR, with the amplicons shown as black rectangles. Scale indicates base pairs relative to the transcription start site (indicated as arrow). The consensus RUNX1 binding motif and the region where RUNX1 was detected in CD34+ and Kasumi-1 cells (Beck *et al.* 2013, Ptasinska *et al.* 2014) are shown. **B-C)** ChIP assays were performed with an antibody against RUNX1 in **A)** KG-1a and **B)** Kasumi-1 cells. Immunoprecipitated DNA was analysed by qPCR using primers described in (A) and in *Section 4.2.2.3*. The data are shown as the ratio of immunoprecipitated DNA to total input DNA. Values are expressed as mean ±SEM (n=6 for KG1a and n=3 for Kasumi-1). Statistical significance was determined using Students' *t* Test with no significant difference ($p>0.05$) detected for data in B). In C), binding was not detected at Distal-1 or Rhodopsin regions.

To determine if the upstream regions can function as enhancers, the regions were cloned together with the ITGB4 promoter and analysed using reporter assays. The 5' end of the potential enhancer, which included -13,845 bp to -14,156 bp (Distal-1), and the 3' end of the potential enhancer, which included -12,077 bp and -12,408 bp (Distal-2), were cloned into the ITGB4 Del-6 construct. The Distal-1 and Distal-2 regions were cloned directly adjacent to the 5' end of the ITGB4 promoter region (-175 bp to +144 bp) in the Del-6 construct to determine if either region had enhancer potential in combination with the ITGB4 promoter (Figure 4.14A). The Del-6 and Distal-Del-6 constructs were transfected into K562 cells and luciferase activity was measured after 24 hours. Interestingly, the Distal-1-Del-6 construct had a significantly higher level of reporter activity compared to the Del-6 construct (Figure 4.14B; $p<0.05$), while the Distal-2-Del-6 construct displayed no change in reporter activity compared to the Del-6 construct (Figure 4.14B).

To determine if the Distal-1 region is responsive to RUNX1, K562 cells were transfected with the Del-6 and Distal-1-Del-6 constructs along with the RUNX1 expression plasmid. Cells were harvested after 24 hours and luciferase activity was measured. Fold change in each construct with RUNX1 overexpressed was analysed. While both reporters were activated by RUNX1, unexpectedly, addition of the distal region did not increase RUNX1 responsiveness compared to the Del-6 region alone (Figure 4.14C).

Taken together, these data suggest that the Distal-1 region can function as an enhancer for the ITGB4 promoter and is enriched for RUNX1 in myeloid cells; however, it does not provide increased activity in response to RUNX1 compared to the promoter alone at least in reporter assays.

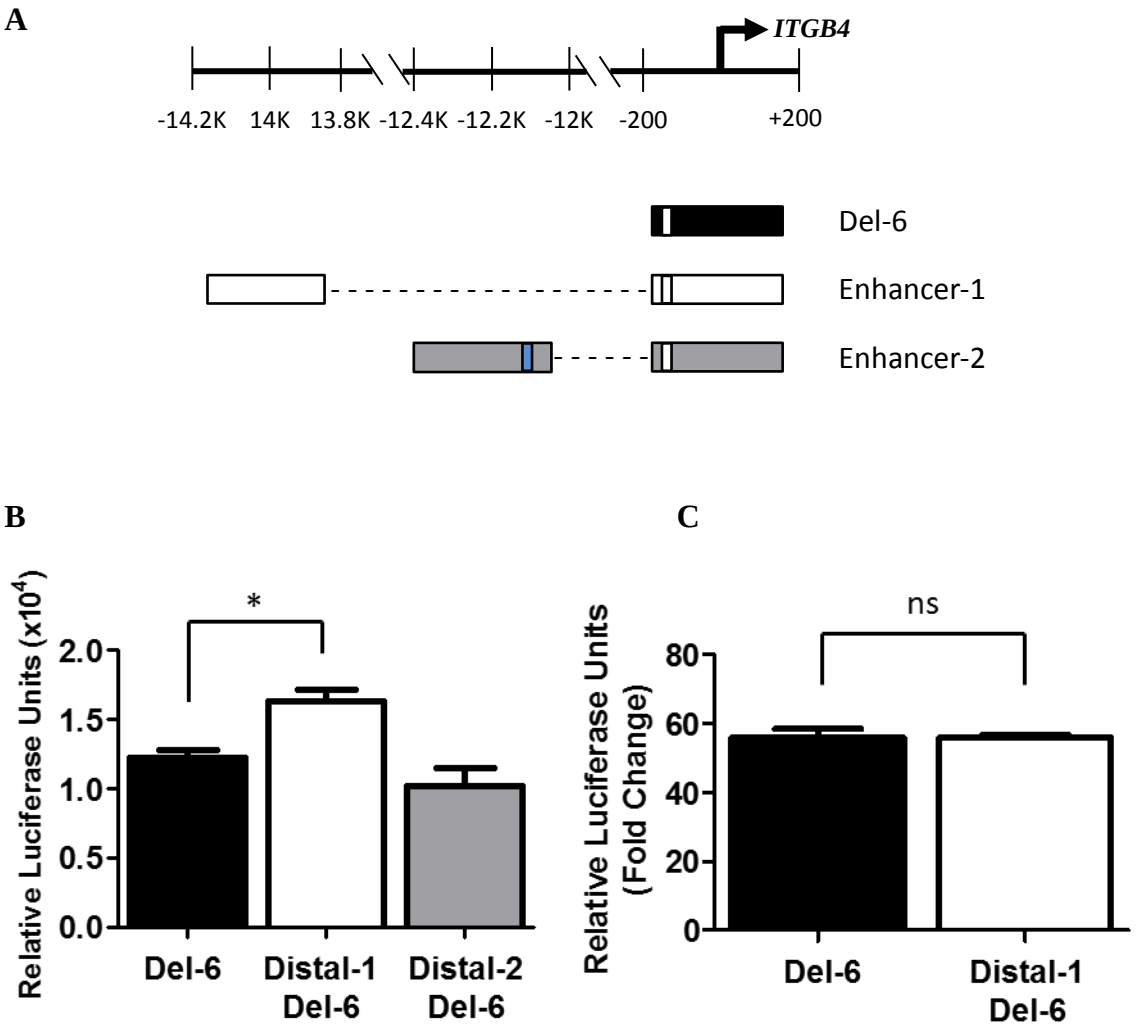


Figure 4.14 – Effects of potential enhancer region on ITGB4 promoter activity. A) Schematic representation of ITGB4 promoter region and distal regions used in reporter assays. Del-6, Distal-1-Del-6 and Distal-2-Del-6 regions were cloned into the pXPG plasmid containing a luciferase gene. Scale indicates base pairs relative to the transcription start site (indicated by arrow). White boxes represent putative RUNX1 binding motifs and the blue box represents a motif with 100% match to the RUNX1 consensus sequence. **B)** Basal level of activity of Del-6, Distal-1-Del-6 and Distal-2-Del-6 constructs following transfection into K562 cells. Protein was isolated from transfected cells after 24 hours and analysed in a luciferase reporter assay. Values are expressed as ±SEM (n=3). Statistical significance was determined using one-way ANOVA, Newman Keuls Multiple Comparison Test, *p<0.05. **C)** K562 cells were transfected with Del-6 and Distal-1-Del-6 constructs either with or without the RUNX1 expression plasmid. Protein was isolated from transfected cells after 24 hours and analysed in a luciferase reporter assay. Fold change in activity of Del-6 and Distal-Del-6 constructs with RUNX1 overexpressed in K562 cells is shown. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using Students' *t* Test, ns p>0.05.

4.3 Discussion

RUNX1 is commonly described as a sequence-specific DNA binding protein which binds to the consensus motif TGT/cGGT through its DNA binding Runt homology domain (Meyers *et al.* 1993). However, it is clear now that RUNX1 can regulate its target genes through a variety of mechanisms due to its ability to act in complexes with other transcription factors, its ability to direct epigenetic modification and higher order chromatin structures, and its ability to regulate genes through both promoters as well as distal regulatory regions such as enhancers (Bowers *et al.* 2010, Elagib *et al.* 2003, Huang *et al.* 2009, Kitabayashi *et al.* 1998, Levantini *et al.* 2011, Reed-Inderbitzin *et al.* 2006, Waltzer *et al.* 2003, Wotton *et al.* 1994, Zhang *et al.* 1996). Data presented here suggest that RUNX1 regulates the ITGA6 and ITGB4 integrin genes via two distinct mechanisms.

In the present study, RUNX1 was shown to regulate the ITGA6 promoter via the classical mechanism, which is commonly described in the literature. Like most described examples of promoters regulated by RUNX1 (Meyers *et al.* 1993, Takahashi *et al.* 1995, Zhang *et al.* 1994), the ITGA6 promoter possesses a consensus RUNX1 binding motif (TGTGGT) and RUNX1 activation of the promoter was dependent on the presence of this consensus motif. Mutation to the site resulted in a significant decrease in the ability of RUNX1 to activate the promoter. RUNX1 was also shown to bind to the endogenous ITGA6 promoter in both KG-1a and Kasumi-1 cells, therefore suggesting that RUNX1 regulates this gene in myeloid cells.

In contrast, data presented here suggest that RUNX1 regulates the ITGB4 gene by an alternate mechanism. While RUNX1 was shown to activate the ITGB4 promoter in reporter assays and bind to the endogenous promoter in KG-1a and Kasumi-1 cells, the promoter did not possess a consensus RUNX1 binding motif within the RUNX1 responsive region located -175 bp to -58 bp upstream of the transcription start site. However, a variant RUNX1 binding motif, which has been identified in a previous study to bind Runx1 (Tanaka *et al.* 2012), is present within the RUNX1 responsive region, but mutation to this site resulted in no significant decrease in RUNX1 activation of the promoter. The data therefore suggest that RUNX1 regulation of ITGB4 is more complex than that of ITGA6, and does not involve direct binding to a RUNX1 consensus motif.

RUNX1 is a relatively weak transcription factor on its own and therefore is often involved in multi-protein complexes assembled between promoters and distal regions to regulate its target genes (Giese *et al.* 1995, Gu *et al.* 2000, Huang *et al.* 2009, Mao *et al.* 1999, Zhang *et al.* 1996). In recent genome-wide studies RUNX1 has been found to frequently co-occupy regions of the genome with other transcription factors such as SCL, LYL1, LMO2, ERG, FLI1, GATA1 and GATA2, which are also important for haematopoiesis (Beck *et al.* 2013, Tijssen *et al.* 2011, Wilson *et al.* 2010). Interestingly, the majority of these regions possess GATA sites (targeted by GATA1 and GATA2) and ETS sites (targeted by ERG and FLI1), while 76% of regions contain E-box sites (targeted by SCL, LYL1 and LMO2) and only 39% contain RUNX consensus motifs (Wilson *et al.* 2010). The lack of RUNX consensus motifs despite RUNX1 occupancy of the DNA suggests that RUNX1 may be recruited indirectly to many regions of DNA in which these other transcription regulators are present, and synergistically regulate target genes in a large multi-transcription factor complex.

Additionally, RUNX1 is often involved in higher order chromatin structures and can regulate its target genes by binding to both promoter and distal regulatory regions (Levantini *et al.* 2011). RUNX1 has been shown to be important to facilitate the interaction between a downstream enhancer and the promoter of the CD34 gene in haematopoietic cells (Levantini *et al.* 2011). RUNX1 was found to bind to a consensus RUNX1 binding motif present in the enhancer and mutation to this site led to disruption of the promoter-enhancer interaction and decreased *CD34* expression (Levantini *et al.* 2011). While RUNX1 binds directly to many enhancer regions through its Runt domain, its subsequent interaction with promoter regions may therefore be through alternative interactions, which do not rely on known RUNX1 motifs.

Since the ITGB4 promoter does not possess any consensus RUNX1 binding motifs and RUNX1 activity on the promoter is not dependent on the presence of any other variant RUNX1 binding motifs, these data suggest that RUNX1 is recruited to the promoter indirectly. To determine if RUNX1 may be recruited to the promoter by other haematopoietic transcription factors such as SCL, LYL1, LMO2, ERG, FLI1, GATA1 and GATA2, the promoter was examined for binding motifs of these transcription factors. Interestingly, a site that is recognised by a LMO2 complex, containing SCL, E2A and GATA1 proteins, is present in the RUNX1 responsive region of the ITGB4 promoter, located at -74 bp to -79 bp upstream of the transcription start site (Figure 4.15A). The site

identified, CAGGTG, is an E-box motif (CANNTG), which is targeted by SCL, E2A, SCL-E2A heterodimers, LYL1 and LYL1-E2A heterodimers (Ellenberger *et al.* 1994, Miyamoto *et al.* 1996, Sanda *et al.* 2012) and the specific sequence present in the ITGB4 promoter has been shown to bind LMO2 complexes containing SCL, E2A and GATA1 proteins (Wadman *et al.* 1997). In addition, an ETS binding motif (GGAA/T), which is targeted by ERG and FLI1 transcription factors (Sementchenko and Watson 2000), is also present in the RUNX1 responsive region of the ITGB4 promoter, located at -57 bp to -61 bp upstream of the transcription start site (Figure 4.15A). The site identified, AGGAA, has been shown to bind ERG and FLI1 transcription factors in a previous ChIP-seq study (Wilson *et al.* 2010). Taken together, this analysis suggest RUNX1 may be recruited to the ITGB4 promoter to form a transcription factor complex with SCL, E2A, GATA1, LYL1, ERG and FLI1 which bind to an E-box site and ETS motif to regulate *ITGB4* expression.

While RUNX1 may be recruited to the ITGB4 promoter indirectly by interacting with other transcription factors present at the promoter, data presented here suggest that RUNX1 may also regulate the ITGB4 promoter through an enhancer located -12,053 bp to -14,003 bp upstream of the transcription start site. The enhancer region was identified to interact with the ITGB4 promoter through ChIA-PET data (ENCODE 2012), which showed a DNA loop between the ITGB4 promoter and enhancer regions coinciding with RNA PolII binding in K562 cells. Additionally, RUNX1 was shown to bind to the 5' end of this region in KG-1a cells in ChIP analysis and this was supported by the ChIP-seq study by Beck *et al*. (2013), which showed RUNX1 binding at this region in CD34+ cells. Additionally, in contrast to this study, RUNX1 was detected at this region in Kasumi-1 cells in the ChIP-seq study by Ptasinska *et al*. (2014). Furthermore, reporter assays demonstrated that the 5' end of the interacting region has enhancer potential due to increased ITGB4 promoter activity in K562 cells. However, unexpectedly, when RUNX1 was overexpressed in the cells, activity of the promoter was not increased. There could be a number of reasons for this, including the failure of the reporter construct, in which the enhancer and promoter are adjacent, to form the type of loop detected in the endogenous cells. Alternatively, RUNX1 may only bind one of the regions endogenously. For example, since the promoter and enhancer regions interact, RUNX1 detected at the enhancer in ChIP analysis, may be due to binding of RUNX1 at the promoter, and vice versa.

Similar to the ITGB4 promoter, while RUNX1 was shown to bind to the ITGB4 enhancer in ChIP analysis, this region does not possess any consensus RUNX1 binding motifs, suggesting that RUNX1 may also be recruited to the enhancer indirectly. To determine if RUNX1 may be recruited to the enhancer by other haematopoietic transcription factors such as SCL, LYL1, LMO2, ERG, FLI1, GATA1 and GATA2, the enhancer was examined for binding motifs of these transcription factors also. Interestingly, an E-box motif (CACCTG), located at -13,842 bp to -13,847 upstream of the transcription start site (Figure 4.15B), which is a binding motif for an E2A protein (E47) (Ellenberger *et al.* 1994), was identified. Furthermore, it has been shown that the E47 protein, in association with the SCL protein, binds to E-box motifs found in eukaryotic enhancer regions (Hsu *et al.* 1991). Also present in the ITGB4 enhancer region is a GATA site, located -14,077 bp to -14,080 bp upstream of the transcription start site (Figure 4.15B), which is a binding motif for GATA1 and GATA2 proteins, and two ETS motifs (AGGAA), located -13,810 bp to -13,814 bp and -13,917 bp to -13,921 bp upstream of the transcription start site (Figure 4.15B), which are binding motifs for ERG and FLI1 proteins (Sementchenko and Watson 2000, Wilson *et al.* 2010). Taken together, this analysis suggest that RUNX1 may also be recruited to the ITGB4 enhancer to form a transcription factor complex with SCL, E2A, GATA1, LYL1, ERG and FLI1 which bind to E-box, ETS and GATA motifs present in the enhancer region.

ITGB4 Promoter

-175 CTAGCCGATCGGGGCGCTGGGCGGGCGCCGCGGGAGCCGCAGCCCTTTCCGGGGGGCGGA

-105 CCCGGCTCCGGCGGCGGCACCCAGCTCCTGCCCCGACAGGTGCGCGCCGCGCGAAGGAA

ITGB4 Enhancer

-14,156 GAGGCGGCAGCTCATTGTTTACAGGCAAGCCCTGCTCCTGGGAGGGCTCCTGCCACCCCA

CCCTTCCTCTGTGTGTTATCTCTGCCCCACAGCAGCCCCTGGGCAGCACCAGTGGCCACT

GGGCTCCCCCCGGGTTGAAACGGGTTTCCCAGACCAGGGGTTCAGAGGAGACTATTTACC

CTTCATGATCTTTGGCGATTCTCCACTGGAGCGGAAGGGCTGTGTGTCAAAAGAAGGAAG

CCAGGCTGTGAAGGGCCGTGTTGCTTTCAGTGGGTGGGCAGAGGTTTAGAAAGGTGGTTC

-13,855 TGAAATGGCACCTGGTACTCTTGTGGGACCTGGGGAATATAAGGAA

Figure 4.15 – E-box, ETS and GATA binding motifs present in the ITGB4 promoter and enhancer regions. DNA sequences containing the ITGB4 promoter and enhancer regions, as well as the base pair number are shown. E-box (pink), ETS (blue) and GATA (green) motifs are highlighted in the sequences.

Interestingly, previous studies have shown the multi-transcription factor complex involving RUNX1, FLI1, GATA2, SCL, LYL1, LMO2, ERG, or a subset of these transcription factors, to be enriched at known HSC enhancers (Beck *et al.* 2013, Tijssen *et al.* 2011). This complex has been identified at the *HHEX*+1 haematopoietic stem/progenitor cell enhancer and ERG +85 enhancer in human haematopoietic stem cells, and enrichment of this transcription factor complex has been associated with active histone marks of enhancer regions (Beck *et al.* 2013). Additionally, GATA2, RUNX1, FLI1 and SCL have been shown to bind the *RUNX1* +23 enhancer in primary human megakaryocytes (Tijssen *et al.* 2011). Furthermore, it has recently been demonstrated that SCL, GATA1, LMO2 and Ldb1 are required for long-range interaction between the β-globin locus control region and active globin genes, and the SCL protein is required for the formation of the chromatin loop between the β-globin locus control region and globin genes (Yun *et al.* 2014). The SCL protein may therefore be essential for the long-range interaction between the ITGB4 promoter and enhancer regions.

To provide some evidence that RUNX1 is recruited to the ITGB4 promoter and enhancer regions by other haematopoietic transcription factors, ChIP-seq data (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Ptasinska *et al.* 2014, Tijssen *et al.* 2011, Trombly *et al.* 2015) described in *Chapter 3* were interrogated for binding of these transcription factors at these regions. Interestingly, ERG was detected at the ITGB4 promoter in K562 cells (Figure 4.16), CD34+ cells, AML mononuclear cells from an individual with t(8;21)-positive AML and in APL blasts from an individual with newly diagnosed t(15;17)-positive AML (Martens *et al.* 2012), and the FLI1 transcription factor was detected at the ITGB4 promoter in the leukaemia cell line U937 (Martens *et al.* 2012). Additionally, ERG was detected at the ITGB4 enhancer in MCF7 cells with RUNX1-ETO and ERG overexpressed (Martens *et al.* 2012), thus suggesting that ERG has the capacity to bind to the enhancer in haematopoietic cells. While ERG and FLI1 were detected at ITGB4 enhancer and/or promoter regions, other haematopoietic transcription factors were not detected at these regions in the ChIP-seq data (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Ptasinska *et al.* 2014, Tijssen *et al.* 2011, Trombly *et al.* 2015), however a previous study has suggested that similar to individual transcription factors, multifactor combinatorial binding is cell-type specific (Tijssen *et al.* 2011). Therefore, a complex containing FLI1, GATA2, SCL, LYL1, LMO2, ERG, E2A and RUNX1 may be cell-type specific, which could be why binding of many of these transcription factors were not detected in the cell types analysed in the ChIP-seq studies.

Alternatively, the exact make up of such a complex may differ between cell types. However, this data does support the hypothesis that ERG and FLI1 may be involved in the regulation of *ITGB4* and may also be involved in the recruitment of RUNX1 to the promoter and enhancer.

In conclusion, RUNX1 was found to regulate the ITGA6 and ITGB4 genes by different mechanisms. Data presented here suggests that RUNX1 regulates the ITGA6 promoter through a consensus RUNX1 binding motif and RUNX1 activation of the promoter is dependent on the presence of this site (Figure 4.17A). In contrast, RUNX1 regulates the ITGB4 promoter indirectly and this may be through the interaction with other haematopoietic transcription factors such as FLI1, GATA1/GATA2, SCL, LYL1, LMO2, ERG and E2A and may also require an upstream enhancer region (Figure 4.17B). While previous studies have demonstrated binding of some of these transcription factors to the ITGB4 promoter and enhancer regions, further studies are required to test this model.

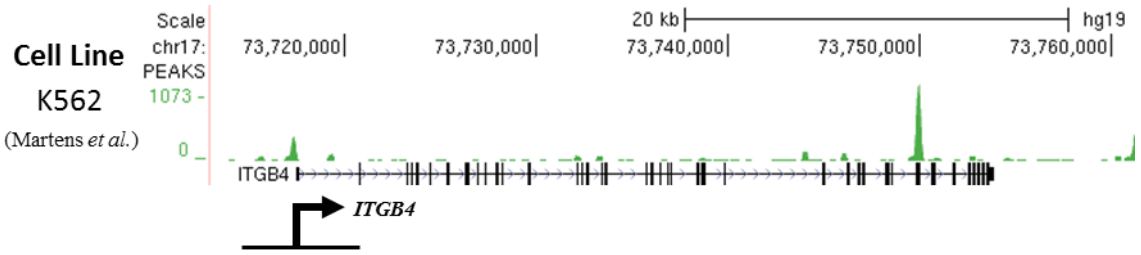
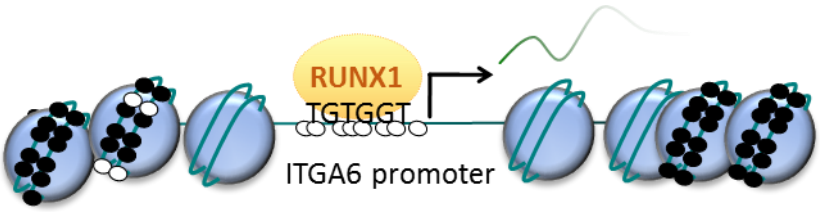


Figure 4.16 – ERG binding at ITGB4 promoter in K562 cells. Data from Martens *et al*. (2012) study was downloaded and visualised using the UCSC Genome Browser (Kent *et al.* 2002). Screen shot was taken from the UCSC browser and show ERG binding across the ITGB4 gene in K562 cells. Peaks represent ERG binding and the black right-angled arrow represents the transcription start site. The ITGB4 gene is represented at the bottom of the figure with exons shown as bars.

A



B

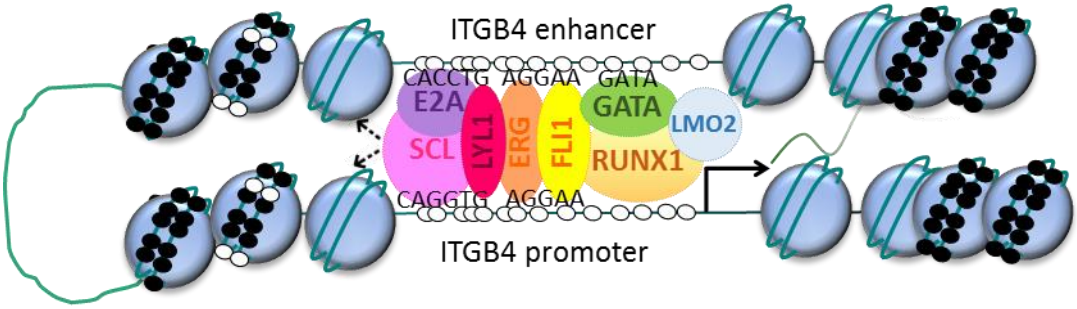


Figure 4.17 – A model for RUNX1 regulation of *ITGA6* and *ITGB4*. A) RUNX1 targets and regulates the ITGA6 promoter through a consensus RUNX1 binding motif. **B)** RUNX1 regulates the ITGB4 promoter indirectly via the interaction with other transcription factors, which bind to E-box motifs (CAGGTG and CACCTG), ETS motifs (AGGAA) and a GATA motif in promoter and/or enhancer regions. The SCL transcription factor is important for the formation of chromatin looping required for the interaction between ITGB4 promoter and enhancer regions (depicted by dotted arrows). DNA is shown as aqua lines wrapped around nucleosomes (blue balls) and CpG sites are shown as small circles either methylated (black) or unmethylated (white). Transcription start sites are shown as black right-angled arrows.

Chapter 5

Epigenetic Regulation of *ITGB4* and *ITGA6*

5.1 Introduction

5.1.1 Regulation of ITGB4 and ITGA6 by Epigenetic Mechanisms

While RUNX1 was found to regulate the ITGB4 and ITGA6 integrin genes, this does not fully explain their differential expression patterns in the leukaemic cell lines K562, KG-1a and Kasumi-1 (Table 5.1) and therefore additional factors must contribute to the differential patterns of expression. This might include other transcription factors that are involved in their expression, as well as epigenetic factors, and there is some evidence that integrin genes, including the ITGB4 and ITGA6 genes, are regulated by epigenetic mechanisms.

Several studies have provided evidence suggesting that *ITGB4* is regulated by DNA methylation. A study by Loss *et al*. (2010) aimed to identify epigenetically regulated genes by comparing gene expression and CpG methylation profiles in 45 breast cancer cell lines. Through this analysis the ITGB4 gene was predicted to be regulated by DNA methylation due to clustering of gene expression and DNA methylation data sets (Loss *et al.* 2010). In support of this, another study found that the DNA methylation status of the Itgb4 promoter was inversely correlated with expression and spatial localisation of β4 protein in the mouse mammary gland (Yang *et al.* 2009). In addition, the ITGB4 gene has been found to be differentially methylated in individuals with diabetes with end stage renal disease compared to individuals with diabetes without neuropathy (Sapienza *et al.* 2011) and in pancreatic islets of individuals with type 2 diabetes compared to non-diabetic controls (Dayeh *et al.* 2014).

In addition to DNA methylation, histone modification has also been shown to influence *ITGB4* expression, as well as the expression of *ITGA6*. Treatment of PC-3 prostate cancer cells with a HDAC inhibitor resulted in decreased expression of both *ITGB4* and *ITGA6* (Hudak et al. 2012, Wedel et al. 2011) and treatment of a hepatocellular carcinoma cell line with a HDAC inhibitor also decreased *ITGA6* expression (Lin *et al.* 2005).

While there is evidence that DNA methylation and/or histone acetylation has an effect on *ITGB4* and *ITGA6* expression, this has not been fully characterised in myeloid cells. Therefore, another aim of this study was to investigate regulation of *ITGB4* and *ITGA6* by epigenetic mechanisms in myeloid cells.

Integrin	Cell Line	Relative Expression
ITGB4	K562	2.2×10^{-4}
	KG-1a	0
	Kasumi-1	5.2×10^{-3}
ITGA6	K562	9.7×10^{-5}
	KG-1a	3.7×10^{-3}
	Kasumi-1	1.7×10^{-3}

Table 5.1 – Relative expression of *ITGB4* and *ITGA6* in leukaemic cell lines. Mean values of expression of ITGB4 and ITGA6 genes in K562, KG-1a and Kasumi-1 cells from RT-qPCR data as depicted in *Figure 3.6A* (Chapter 3). Number of copies of ITGB4/ITGA6 mRNA relative to GAPDH are shown (n=3).

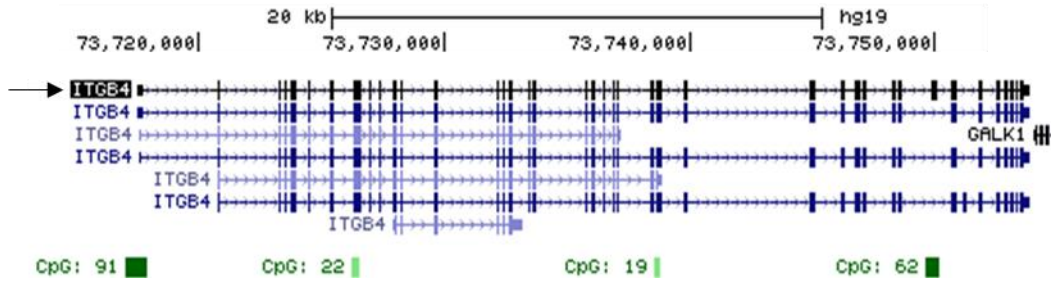
5.2 Results

5.2.1 Identification of a CpG Island at the ITGB4 and ITGA6 Promoters

Approximately 60% of human gene promoters are encompassed by CpG islands and are therefore subject to DNA methylation. To determine if *ITGB4* and *ITGA6* may be regulated by DNA methylation, genome-wide CpG island data from the ENCODE project (ENCODE 2012) were interrogated for the presence of a CpG island at the promoters of the ITGB4 and ITGA6 genes.

Both ITGB4 and ITGA6 genes were found to possess large CpG islands of 824 bp and 1084 bp, respectively, which encompass the transcription start site of the genes (Figure 5.1). The CpG island at the ITGB4 promoter is located -642 bp to +482 bp relative to the transcription start site with a CpG content of 22.1%, and the CpG island at the ITGA6 gene is located -364 bp to +1020 bp relative to the transcription start site with a CpG content of 21.2%. The ITGB4 gene also has three other smaller CpG islands located along the gene body, as shown in *Figure 5.1A.*

A



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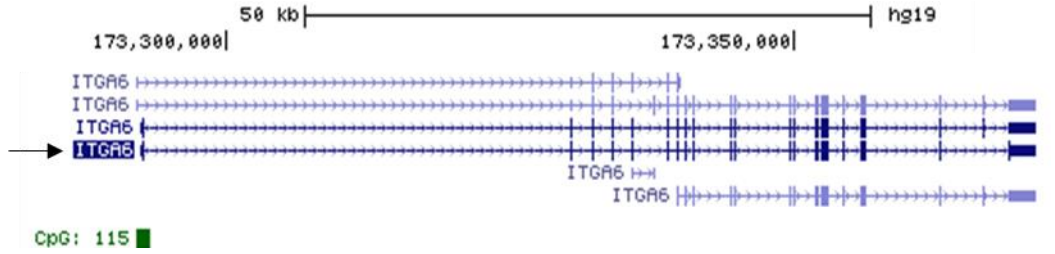


Figure 5.1 – CpG islands along *ITGB4* and *ITGA6*. A screen shot of genome-wide CpG island data (ENCODE 2012) visualised in the UCSC Genome Browser (Kent *et al.* 2002) is shown. CpG islands along **A)** ITGB4 and **B)** ITGA6 genes are shown as green boxes. The numbers adjacent to the boxes represent the CpG count for the particular island. Dark green boxes are regions greater than 300 bp while light green boxes are regions less than 300 bp. All transcript variants for each integrin gene are shown; the major transcript for each gene is shown by a black arrow.

5.2.2 Effect of DNA Methylation and Histone Acetylation on ITGB4 and ITGA6 Expression

To determine if expression of the ITGB4 or ITGA6 genes is influenced by the DNA methylation or histone acetylation status of the gene, their expression was examined in myeloid cells following treatment with epigenetic inhibitors. K562, KG-1a and Kasumi-1 cells were treated with either 500 ng/mL of DAC for 72 hours, 200 ng/mL of TSA for 6 hours or a combined treatment of DAC and TSA. Changes in *ITGB4* and *ITGA6* expression were analysed using RT-qPCR. DAC treatment increased expression of *ITGB4* in all cell lines examined (Figure 5.2A-C), with the fold change in *ITGB4* expression higher in K562 and KG-1a cells (Figure 5.2D). TSA also increased *ITGB4* expression in all cell lines and *ITGB4* expression was increased further when TSA was used in combination with DAC in KG-1a cells (Figure 5.2A-C). In contrast, *ITGA6* expression only increased following DAC treatment in K562 cells (Figure 5.3A), with a 5-fold change in gene expression compared to the untreated cells (Figure 5.3D, $p<0.05$). TSA treatment had no effect on *ITGA6* expression in any of the leukaemic cell lines, either alone or in combination with DAC (Figure 5.3A-C).

Taken together, these data suggest that both ITGB4 and ITGA6 integrin gene expression can be enhanced by treatment with a DNA methylation inhibitor, although this was only apparent for *ITGA6* in K562 cells. However, only ITGB4 gene expression was responsive to a histone deacetylase inhibitor.

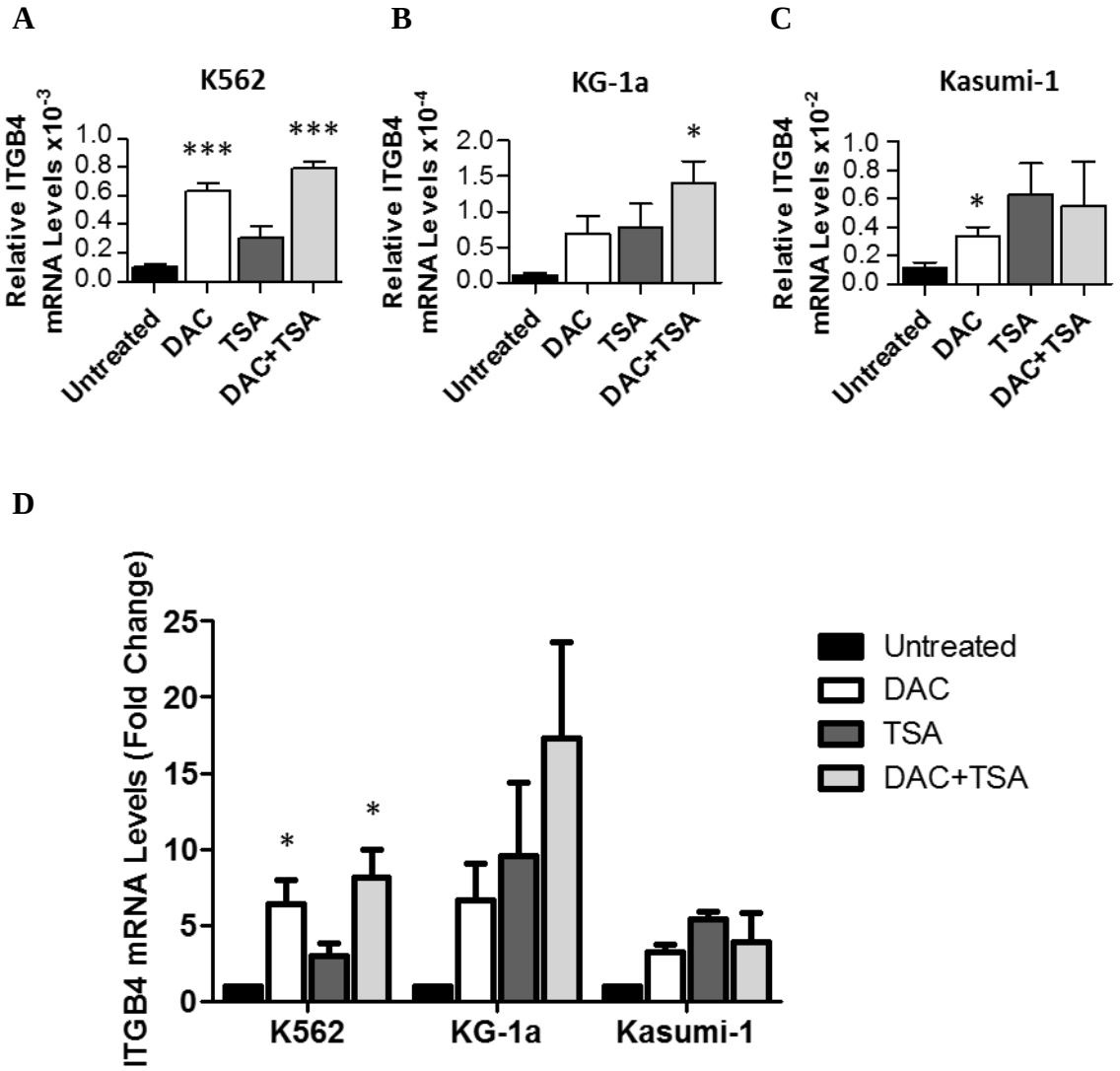


Figure 5.2 – Effects of DAC and TSA treatment on *ITGB4* expression in leukaemic cell lines. Total mRNA was isolated from untreated and treated **A)** K562, **B)** KG-1a and **C)** Kasumi-1 cell lines, reversed transcribed and ITGB4 mRNA levels were analysed using RT-qPCR. Copy number was normalised to β2-Microglobulin and values are expressed as mean ±SEM (n=3). Statistical significance of treated versus untreated cells was determined using Student's *t* Test, ***$p<0.001$, *$p<0.05$. **D)** Data in (A-C) represented as fold change in ITGB4 mRNA levels relative to untreated cells.

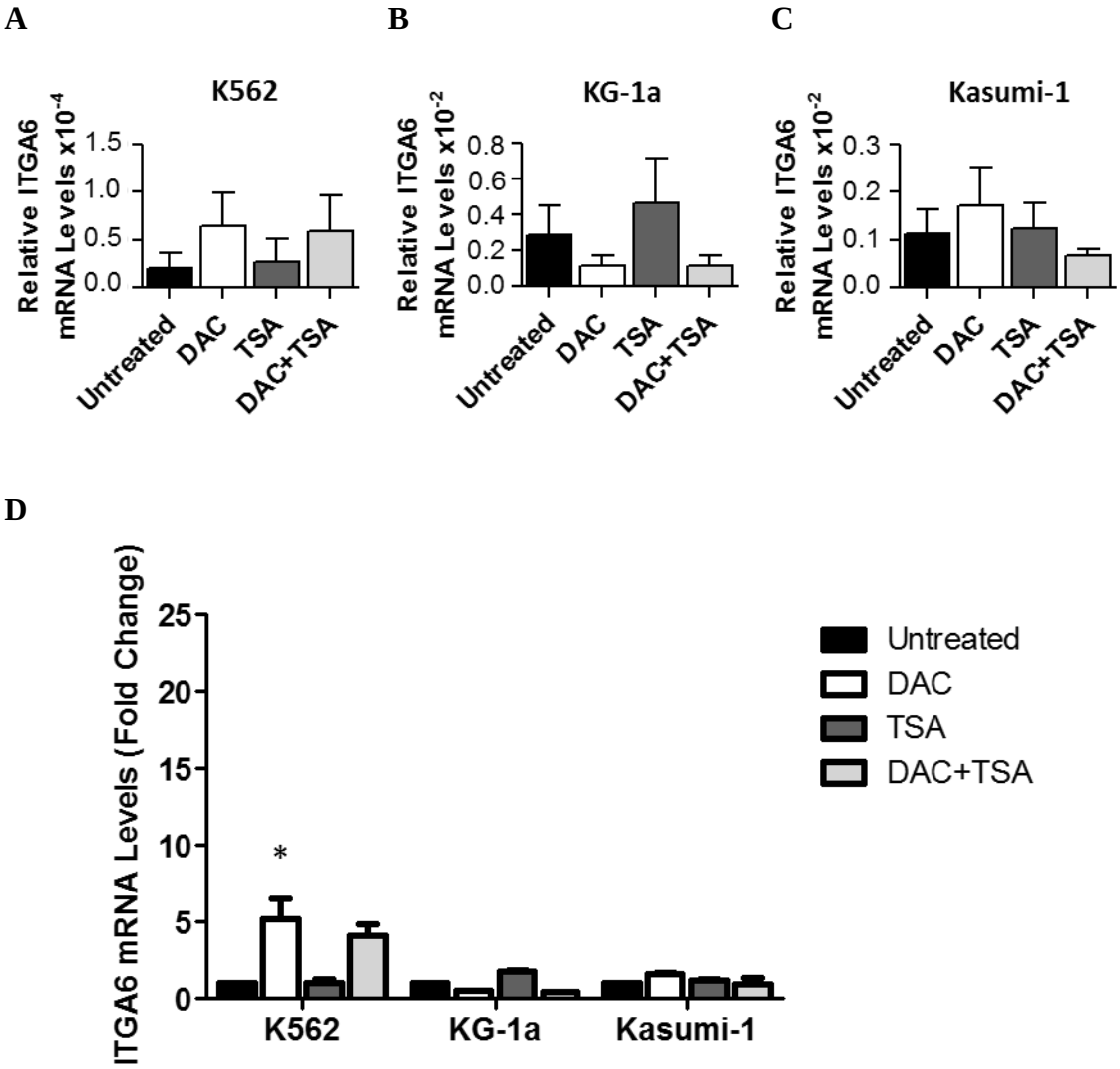


Figure 5.3 – Effects of DAC and TSA treatment on *ITGA6* expression in leukaemic cell lines. Total mRNA was isolated from untreated and treated **A)** K562, **B)** KG-1a and **C)** Kasumi-1 cell lines, reversed transcribed and ITGA6 mRNA levels were analysed using RT-qPCR. Copy number was normalised to β2-Microglobulin and values are expressed as mean ±SEM (n=3). Statistical significance for treated versus untreated cells was determined using Student's *t* Test, **p*<0.05. **D)** Data in (A-C) represented as fold change in ITGA6 mRNA levels relative to untreated cells are shown.

5.2.3 DNA Methylation of ITGB4 and ITGA6 Promoters in Myeloid Cells

Since both *ITGB4* and *ITGA6* responded to DAC treatment, at least in some cell lines, their expression may therefore be regulated by promoter methylation. To determine if the CpG islands located at the ITGB4 and ITGA6 promoters are differentially methylated in the leukaemic cell lines, which express these genes at different levels (Table 5.1), DNA methylation was analysed in K562, KG-1a and Kasumi-1 cells using bisulphite sequencing.

Genomic DNA was isolated from K562, KG-1a and Kasumi-1 cells and was subjected to bisulphite conversion, as described in *Chapter 2, Section 2.6*. PCR primers were designed to amplify the region -516 bp to +276 bp of *ITGB4*, encompassing 85 CpG sites. PCR products were cloned into the pGEM-T Easy vector and single clones were selected for sequencing. Interestingly, the ITGB4 promoter was found to be differentially methylated in the cell lines examined. The promoter was highly methylated in KG-1a cells, but was relatively unmethylated in K562 and Kasumi-1 cells (Figure 5.4). The DNA methylation pattern in KG-1a cells was striking, while there was some methylation upstream of the transcription start site (including through the RUNX1 responsive region), the region downstream of the transcription start site was completely methylated (Figure 5.4).

Taken together, these data suggest that in both K562 and Kasumi-1 cells the ITGB4 promoter is likely to be relatively accessible, whereas the ITGB4 promoter in KG-1a cells is likely to be less accessible due to higher levels of DNA methylation. DNA methylation is generally associated with gene silencing, and therefore the methylation patterns observed at the promoter in KG-1a and Kasumi-1 cells reflects the expression of *ITGB4* in these cells (ie. expression in Kasumi-1 but not KG-1a), whereas in K562 cells the ITGB4 promoter is largely unmethylated which does not explain the low expression of *ITGB4* in these cells (Table 5.1).

To determine how the methylation pattern of the ITGB4 promoter in leukaemic cell lines compares to human samples, DNA methylation of the ITGB4 promoter was analysed in whole blood samples from individuals with (LK7770) and without leukaemia (LK004 and LK124). Interestingly, the ITGB4 promoter was relatively unmethylated in all samples (Figure 5.5) and most similar to the pattern seen in Kasumi-1 cells. While the leukaemic sample, which is positive for the t(8;21) chromosomal translocation, showed

a similar methylation pattern to the t(8;21)-positive Kasumi-1 cell lines, with most methylation located at the 3' end (Figure 5.5), this pattern was also seen in the non-leukaemic cells. These data therefore suggest that the ITGB4 promoter is normally unmethylated in human blood cells, and the presence of the RUNX1-ETO fusion protein does not appear to have an effect on the DNA methylation pattern at the ITGB4 promoter.

DNA methylation of the ITGB4 promoter was also analysed in treated and untreated cell lines to determine if DAC caused demethylation at this region. K562, KG-1a and Kasumi-1 cells were treated with DAC for 72 hours. Genomic DNA was isolated and subjected to bisulphite conversion, as described in *Chapter 2, Section 2.6*. Due to difficulty in amplifying the region -516 bp to +276 bp from DAC treated cells, a different set of PCR primers were used to amplify a smaller region of the ITGB4 CpG island, located -390 bp to +276 bp of *ITGB4*, encompassing 72 CpG sites. The PCR products were cloned into the pGEM-T Easy vector and single clones were sequenced. Treatment of cells with DAC had no effect in K562 cells, which is unsurprising because the ITGB4 CpG island is unmethylated in these cells (Figure 5.6). Kasumi-1 cells displayed demethylation at the 3' end, where most of the methylation is observed (Figure 5.6). Surprisingly, DAC treatment had no effect on DNA methylation of the ITGB4 promoter in KG-1a cells, although this region is heavily methylated in these cells (Figure 5.6).

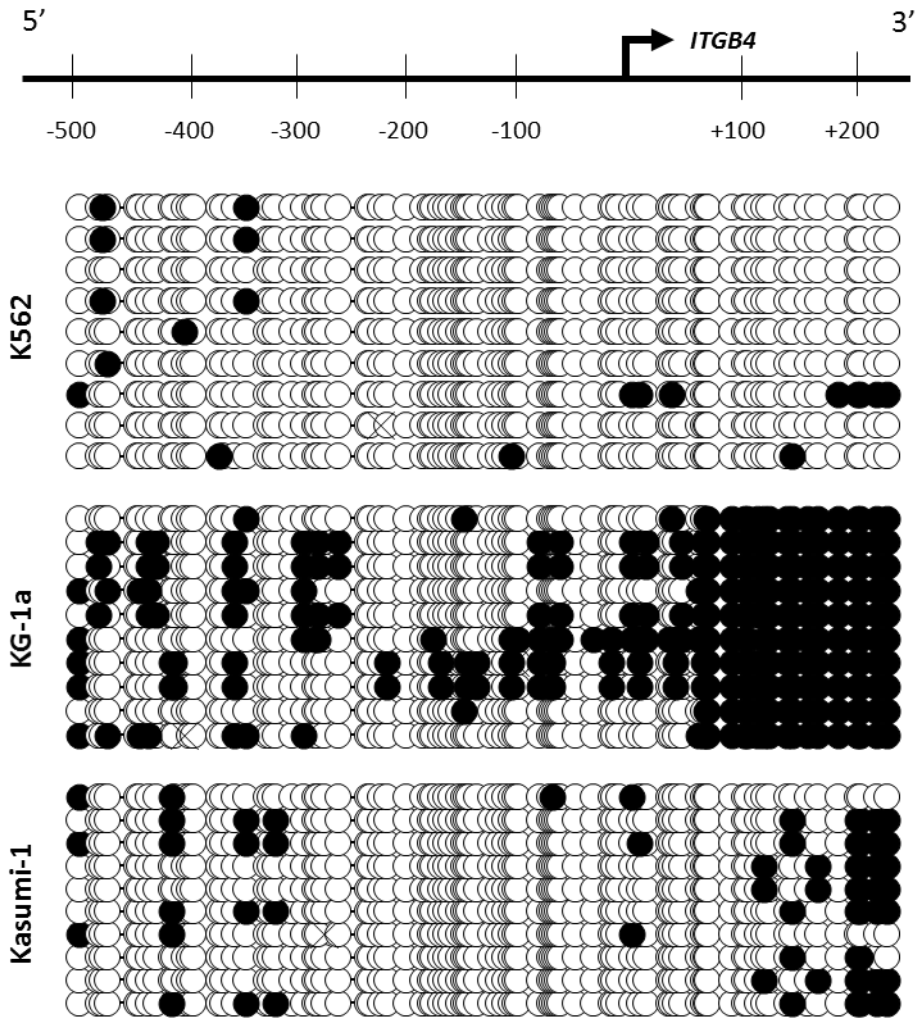


Figure 5.4 – DNA methylation patterns at the ITGB4 CpG island in leukaemic cell lines. CpG sites located in a region of -516 bp to +276 bp of *ITGB4* within the ITGB4 promoter CpG island were analysed for methylation in K562, KG-1a and Kasumi-1 cell lines using bisulphite sequencing. Sequencing was analysed using BiQ Analyzer software and bubble maps were generated using CpG Bubble Chart Generator, Version 20061209 (created by Mark A. Miranda). Each line represents a single clone and circles represent CpG sites. White circles represent unmethylated CpG sites whereas black circles represent methylated sites and crosses are CpG sites with a mutation. Schematic representation of the ITGB4 promoter is shown above and the scale represents base pairs relative to the transcription start site (indicated by arrow).

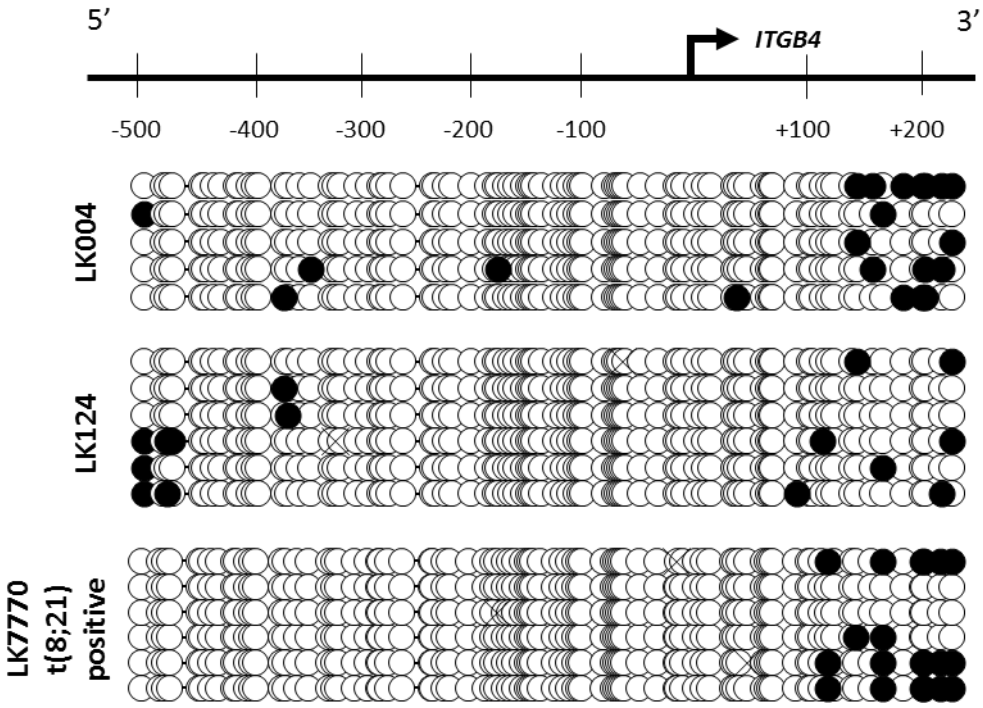


Figure 5.5 – DNA methylation patterns at the ITGB4 CpG island in t(8;21)-positive leukaemia and non-leukaemia samples. CpG sites located in a region of -516 bp to +276 bp of *ITGB4* within the ITGB4 promoter CpG island were analysed for methylation in non-leukaemia (LK004 and LK124) and t(8;21)-positive leukaemia individual samples (LK7770) using bisulphite sequencing. Sequencing was analysed using BiQ Analyzer software and bubble maps were generated using CpG Bubble Chart Generator, Version 20061209 (created by Mark A. Miranda). Each line represents a single clone and circles represent CpG sites. White circles represent unmethylated CpG sites whereas black circles represent methylated sites and crosses are CpG sites with a mutation. Schematic representation of the ITGB4 promoter is shown above and the scale represents base pairs relative to the transcription start site (indicated by arrow).

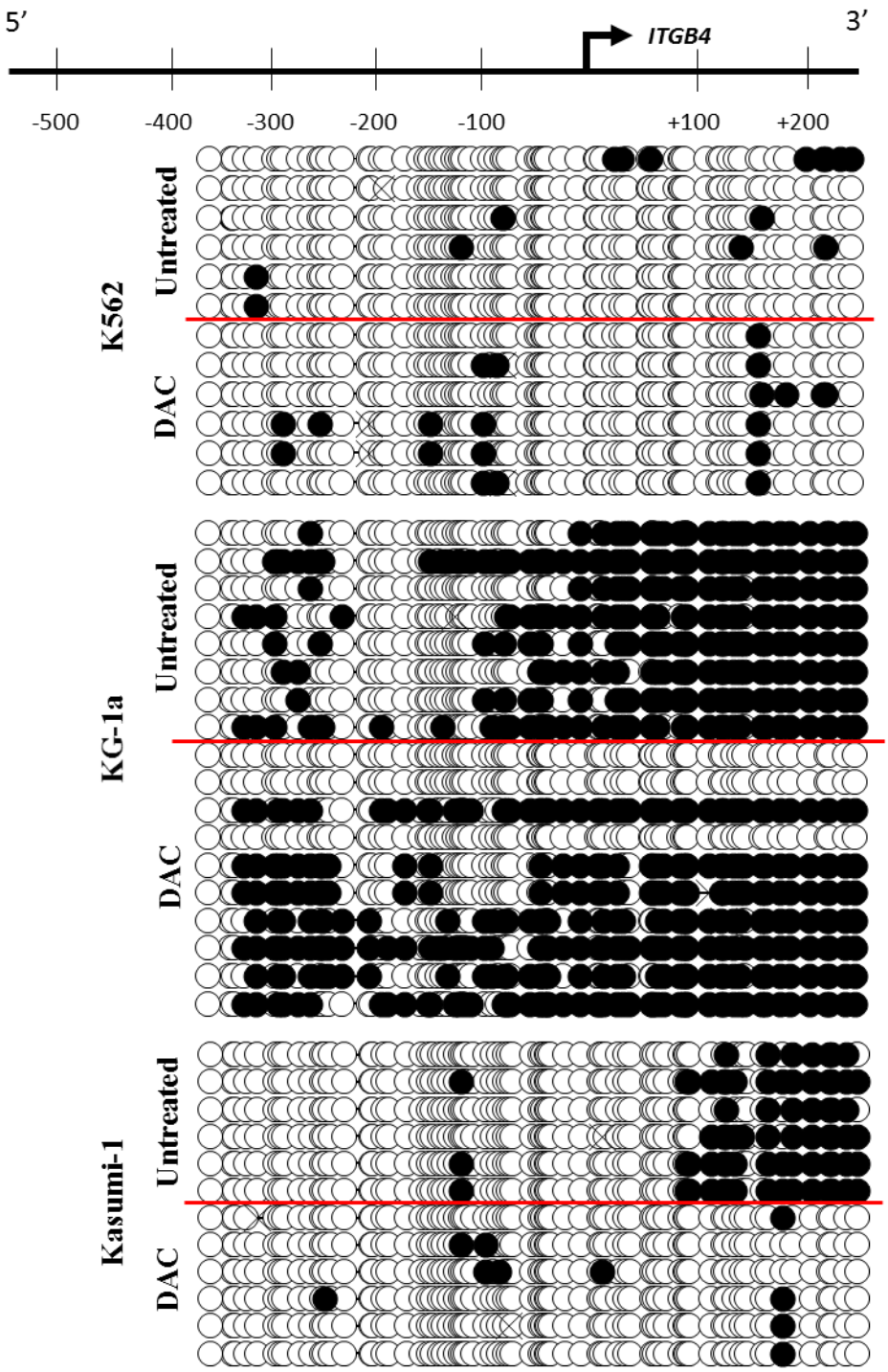


Figure 5.6 - DNA methylation patterns at the ITGB4 CpG island in untreated and DAC treated leukaemic cell lines. CpG sites located in a region of -390 bp to +276 bp of *ITGB4* within the ITGB4 promoter CpG island were analysed for methylation in untreated and DAC treated K562, KG-1a and Kasumi-1 cell lines using bisulphite sequencing. Sequencing was analysed using BiQ Analyzer software and bubble maps were generated using CpG Bubble Chart Generator, Version 20061209 (created by Mark A. Miranda). Each line represents a single clone and circles represent CpG sites. White circles represent unmethylated CpG sites whereas black circles represent methylated sites and crosses are CpG sites with a mutation. Schematic representation of the ITGB4 promoter is shown above and the scale represents base pairs relative to the transcription start site (indicated by arrow).

To determine if the ITGA6 promoter CpG island, similar to the ITGB4 promoter CpG island, is differentially methylated in the leukaemic cell lines and if DAC treatment causes demethylation of the CpG island, bisulphite sequencing was similarly used to analyse CpG methylation at the promoter. K562, KG-1a and Kasumi-1 cells were treated with DAC for 72 hours. Genomic DNA was isolated from treated and untreated cells, and subjected to bisulphite conversion, as described in *Chapter 2, Section 2.6*. PCR primers were designed to amplify the region surrounding the ITGA6 transcription start site in two sections located -329 bp to +240 bp (Fragment A) and +216 bp to +821 bp (Fragment B). PCR products were cloned in the pGEM-T easy vector and single clones were selected for sequencing.

Fragment A, which incorporates the transcription start site of *ITGA6*, was largely unmethylated in all leukaemic cell lines analysed (Figure 5.7). Not surprisingly then, DAC treatment did not result in any significant decrease in methylation at this region (Figure 5.7). Similarly, Fragment B, which incorporates the gene body adjacent to the transcription start site, including the translation start site, was largely unmethylated, although there was some methylation towards the 3' end of the region in K562 cells (Figure 5.8). Similar to Fragment A, treatment with DAC had little effect on methylation of Fragment B due to the existing low levels of methylation (Figure 5.8).

Taken together, these data suggest that the ITGA6 promoter is likely to be relatively accessible in K562, KG-1a and Kasumi-1 cell lines due to low levels of DNA methylation. Unmethylated promoters are typically associated with actively transcribed genes, and therefore the methylation patterns observed at the ITGA6 promoter in KG-1a and Kasumi-1 cells reflects expression of *ITGA6* in these cells (ie. expressed in both cell lines), whereas in K562 cells, the ITGA6 promoter is largely unmethylated which does not explain the low expression of *ITGA6* in these cells (Table 5.1).

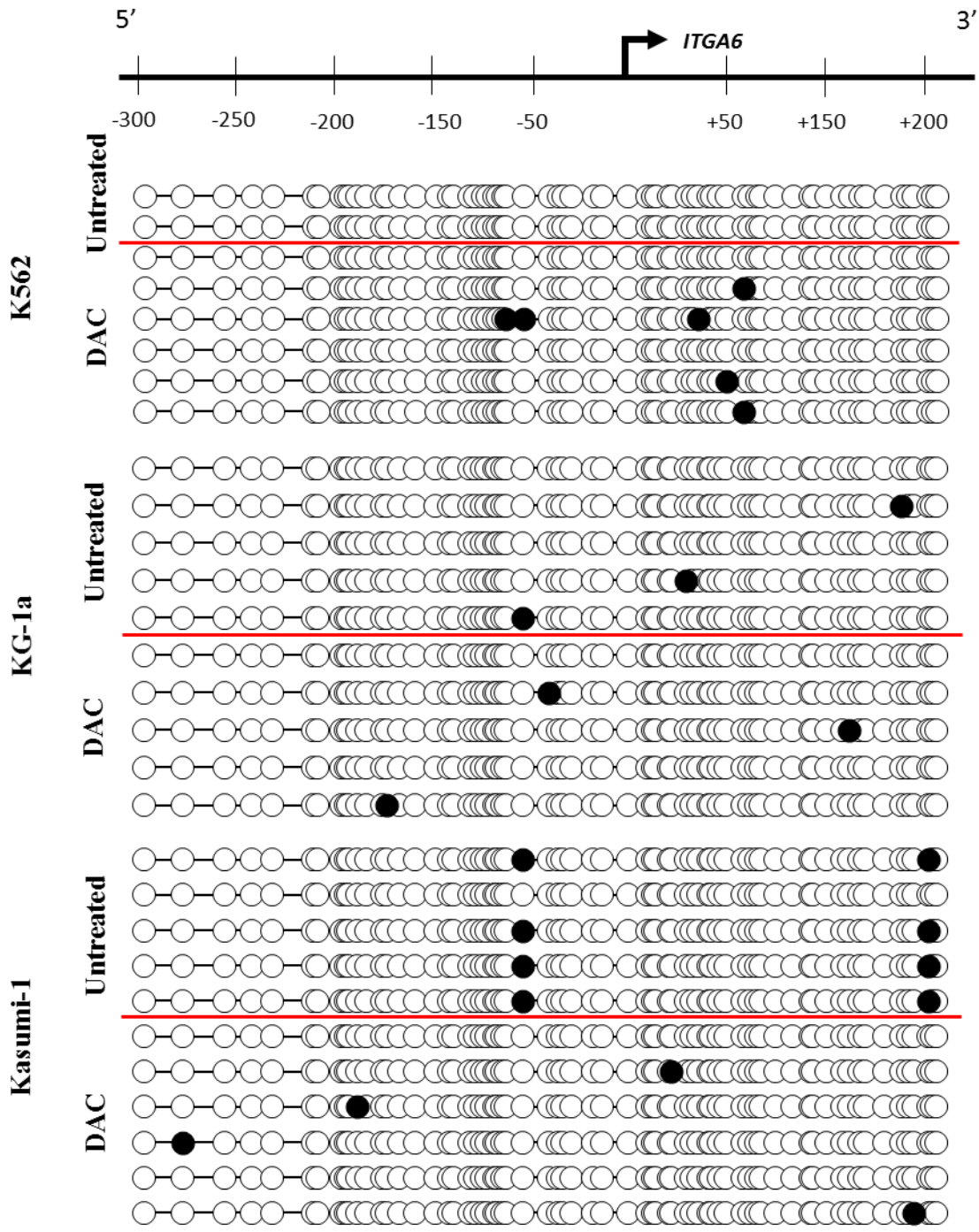


Figure 5.7 – DNA methylation patterns of Fragment A at the ITGA6 CpG island in untreated and DAC treated leukaemic cell lines. CpG sites located in a region of -329 bp to +240 bp of *ITGA6* within the ITGA6 promoter CpG island were analysed for methylation in untreated and DAC treated K562, KG-1a and Kasumi-1 cell lines using bisulphite sequencing. Sequencing was analysed using BiQ Analyzer software and bubble maps were generated using CpG Bubble Chart Generator, Version 20061209 (created by Mark A. Miranda). Each line represents a single clone and circles represent CpG sites. White circles represent unmethylated CpG sites whereas black circles represent methylated sites and crosses are CpG sites with a mutation. Schematic representation of the ITGA6 promoter is shown above and the scale represents base pairs relative to the transcription start site (indicated by arrow).

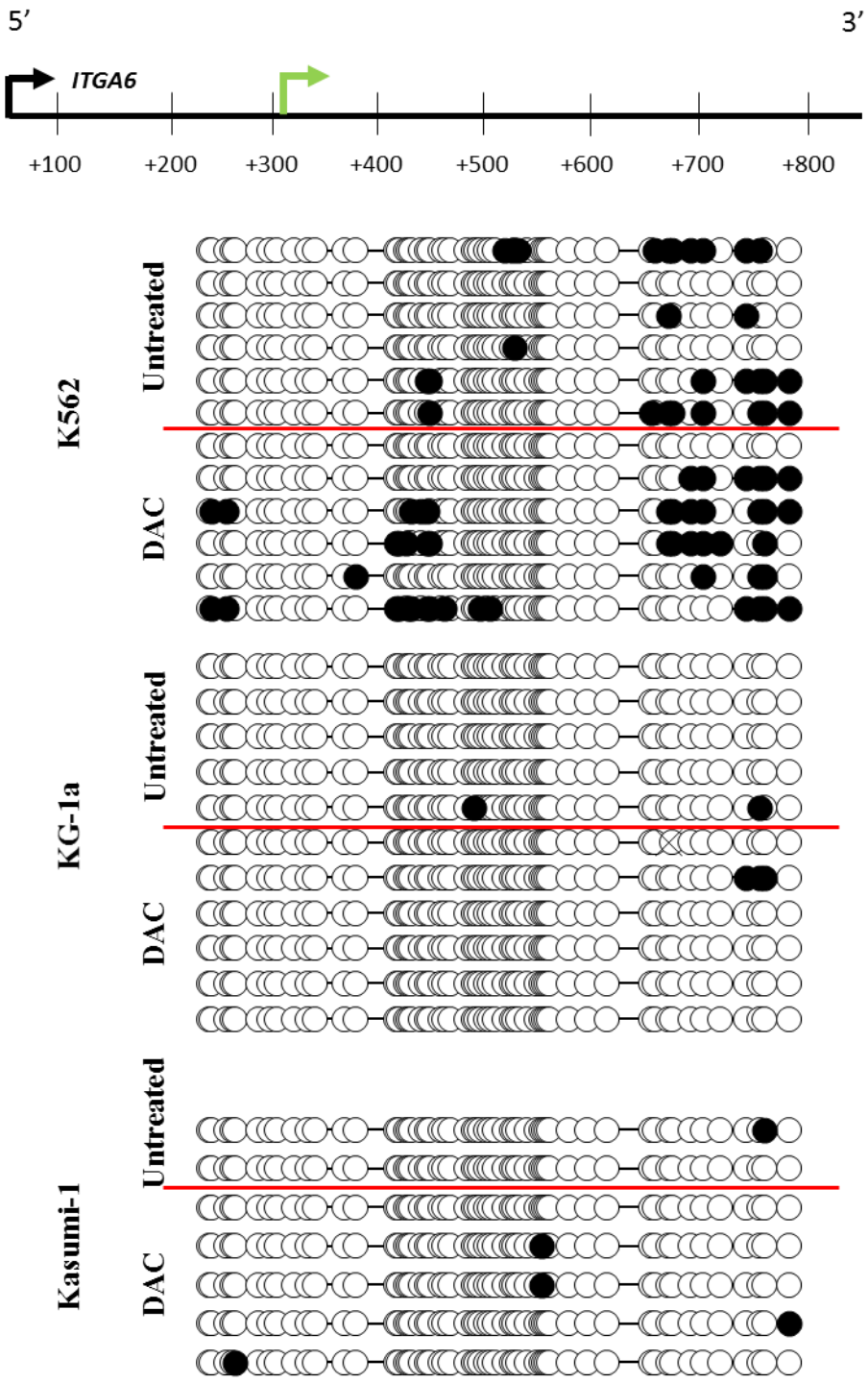


Figure 5.8 – DNA methylation patterns of Fragment B at the ITGA6 CpG island in untreated and DAC treated leukaemic cell lines. CpG sites located in a region of +216 bp to +821 bp of *ITGA6* within the ITGA6 promoter CpG island were analysed for methylation in untreated and DAC treated K562, KG-1a and Kasumi-1 cell lines using bisulphite sequencing. Sequencing was analysed using BiQ Analyzer software and bubble maps were generated using CpG Bubble Chart Generator, Version 20061209 (created by Mark A. Miranda). Each line represents a single clone and circles represent CpG sites. White circles represent unmethylated CpG sites whereas black circles represent methylated sites and crosses are CpG sites with a mutation. Schematic representation of the ITGA6 promoter is shown above and the scale represents base pairs relative to the transcription start site (indicated by black arrow). Translation start site is indicated by the green arrow.

5.2.4 Histone Occupancy at the ITGB4 and ITGA6 Promoters in KG-1a and Kasumi-1 Cells

The positioning of nucleosomes throughout the genome plays a key role in the regulation of gene expression. For most genes there is a well-positioned nucleosome located upstream of the transcription start site, covering a region -300 bp to -150 bp of the transcription start site, which regulates the accessibility of the promoter to regulatory proteins (Jiang and Pugh 2009). When transcription occurs, this nucleosome often undergoes changes which can include histone replacement, histone acetylation and methylation, repositioning of the histone and removal of the histone from the DNA (Jiang and Pugh 2009). Promoters of active genes are often more accessible to regulatory proteins due to decreased histone occupancy.

Since ITGB4 and ITGA6 promoter DNA methylation reflected expression of these genes in KG-1a and Kasumi-1 cells, these promoters may also be affected by other epigenetic mechanisms in these cells. To determine if histone occupancy reflects *ITGB4* and *ITGA6* expression in KG-1a and Kasumi-1 cells, ChIP assays were used to analyse histone H3 occupancy at the integrin promoters. KG-1a and Kasumi-1 cells were fixed with formaldehyde and DNA was sheared using sonication. DNA immunoprecipitated with a histone H3 antibody was analysed by qPCR with primers designed to amplify promoter regions of *ITGB4* and *ITGA6*. As a control, histone H3 occupancy was also measured at the inactive Rhodopsin promoter.

Histone H3 occupancy at the ITGA6 promoter was similar between the two cell lines and was lower than the histone H3 occupancy measured at the silenced Rhodopsin gene, therefore reflecting *ITGA6* expression in these cells (Figure 5.9). In contrast, histone H3 occupancy at the ITGB4 promoter in KG-1a cells was similar to levels seen at the Rhodopsin promoter, which may reflect low levels of *ITGB4* expression in these cells. In contrast, lower histone H3 occupancy was detected at the ITGB4 promoter in Kasumi-1 cells, which was similar to levels observed for the ITGA6 promoter and reflects higher *ITGB4* expression detected in these cells (Figure 5.9).

Taken together, these data show differences in histone H3 occupancy at the integrin gene promoters, which is inversely correlated with expression of these genes in the leukaemic cell lines KG-1a, and Kasumi-1.

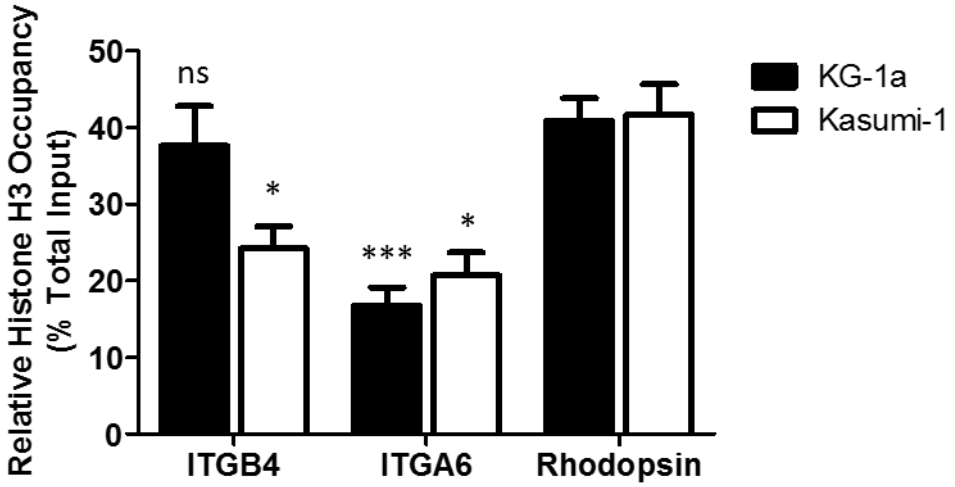


Figure 5.9 – Histone H3 occupancy of ITGB4 and ITGA6 promoters in KG-1a and Kasumi-1 cells. ChIP assays were performed in KG-1a and Kasumi-1 cells with an antibody against histone H3. Immunoprecipitated DNA was analysed by qPCR with primers, which amplify a region of ITGB4, ITGA6 and Rhodopsin promoters. The data are shown as the ratio of immunoprecipitated DNA to total input DNA. Values are expressed as mean ±SEM (n=3-6). Statistical significance was determined using Students' *t* Test, comparing occupancy at integrin promoter regions to the Rhodopsin promoter, ***$p<0.001$, *$p<0.05$, ns $p>0.05$. No significant difference in histone occupancy was detected between cell lines at any region tested.

5.2.5 Histone Acetylation of the ITGB4 and ITGA6 Promoters in KG-1a Cells

Histone acetylation is strongly associated with transcriptional activation of genes (Virani *et al.* 2012). It is thought to enhance transcription through disrupting the interaction between the negatively charged DNA and the positively charge histones by neutralising the charge on the histone proteins (Virani *et al.* 2012). In KG-1a cells, *ITGB4* and *ITGA6* have different DNA methylation patterns and histone H3 occupancy that reflects their different expression levels in these cells. Therefore, to determine if different levels of histone acetylation also reflect expression of these genes in KG-1a cells, ChIP assays were used to determined histone acetylation at the integrin promoters. KG-1a cells were fixed with formaldehyde and DNA was sheared using sonication. DNA immunoprecipitated with an acetyl-H3 antibody was analysed by qPCR with primers designed to amplify promoter regions of *ITGB4* and *ITGA6*. Again, as a control, histone acetylation was also measured at the inactive Rhodopsin promoter. As expected, there was enrichment of acetylation of histone H3 at the promoter of *ITGA6*, compared to the ITGB4 promoter, which was at a similar level to the silenced Rhodopsin promoter (Figure 5.10).

Taken together, these data suggest that expression of *ITGA6* in KG-1a cells is facilitated by low levels of DNA methylation and histone H3 occupancy, and higher levels of histone acetylation at the promoter; whereas low expression of *ITGB4* in KG-1a cells is in part due to higher levels of DNA methylation and histone H3 occupancy, and lower levels of histone acetylation at the promoter.

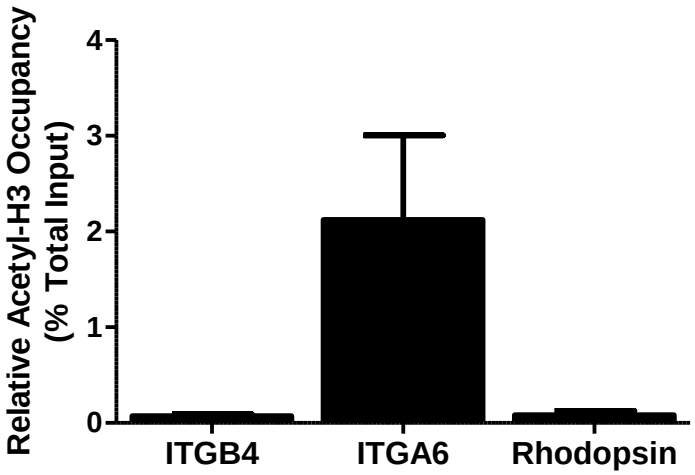


Figure 5.10 – Histone H3 acetylation of ITGB4 and ITGA6 promoters in KG-1a cells. ChIP assays were performed in KG-1a cells with an antibody against acetyl-H3. Immunoprecipitated DNA was analysed by qPCR with primers that amplify a region of ITGB4, ITGA6 and Rhodopsin promoters. The data are shown as the ratio of immunoprecipitated DNA to total input DNA. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using Students' *t* Test, with no significant difference detected ($p>0.05$).

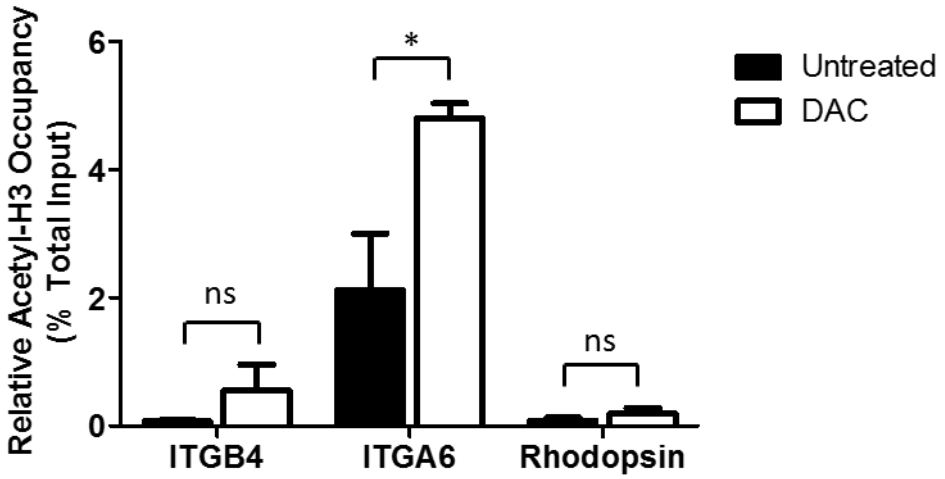
5.2.6 Effect of DAC on Histone Occupancy, Histone Acetylation and RUNX1 Binding at the ITGB4 and ITGA6 Promoters in KG-1a Cells

Cells treated with DAC undergo genome-wide changes which causes global demethylation and activation of many genes, including tumour suppressor genes (Momparler *et al.* 2014). While DAC treatment had no effect on DNA methylation at the ITGB4 promoter in KG-1a cells, it increased expression of *ITGB4* in these cells (Figure 5.2). Therefore, to investigate how DAC is influencing expression, effects on histone H3 occupancy, histone acetylation and/or RUNX1 binding at the ITGB4 promoter in KG-1a cells were examined. Additionally, the ITGA6 promoter was examined to determine the effects of DAC on a gene that is normally expressed in the cells. KG-1a cells were treated with DAC for 72 hours. Untreated and treated cells were fixed with formaldehyde and DNA was sheared using sonication. DNA immunoprecipitated with histone H3, acetyl-H3 and RUNX1 antibodies was analysed by qPCR with primers designed to amplify promoter regions of *ITGB4* and *ITGA6*. Again, as a control, occupancy of histone H3, acetyl-H3 and RUNX1 was also analysed at the inactive Rhodopsin promoter.

Treatment of KG-1a cells with DAC resulted in increased histone acetylation at both the ITGB4 and ITGA6 promoters, reaching statistical significance for ITGA6 (Figure 5.11A; $p<0.05$). As seen before, histone H3 levels were higher at the ITGB4 promoter compared to the ITGA6 promoter, but DAC treatment had no effect on histone H3 occupancy at either ITGB4 or ITGA6 promoter regions (Figure 5.11B). Interestingly, DAC treatment caused a trend in increased RUNX1 binding at the ITGB4 promoter, while RUNX1 binding decreased at the ITGA6 promoter in DAC treated KG-1a cells (Figure 5.12).

Taken together, these data suggest that while DAC treatment does not directly affect DNA methylation at the ITGB4 and ITGA6 promoters, it leads to increased histone acetylation at ITGB4 and ITGA6 promoters in KG-1a cells. The increase in *ITGB4* expression observed in DAC treated KG-1a cells may therefore be due to increased histone acetylation at the promoter.

A



B

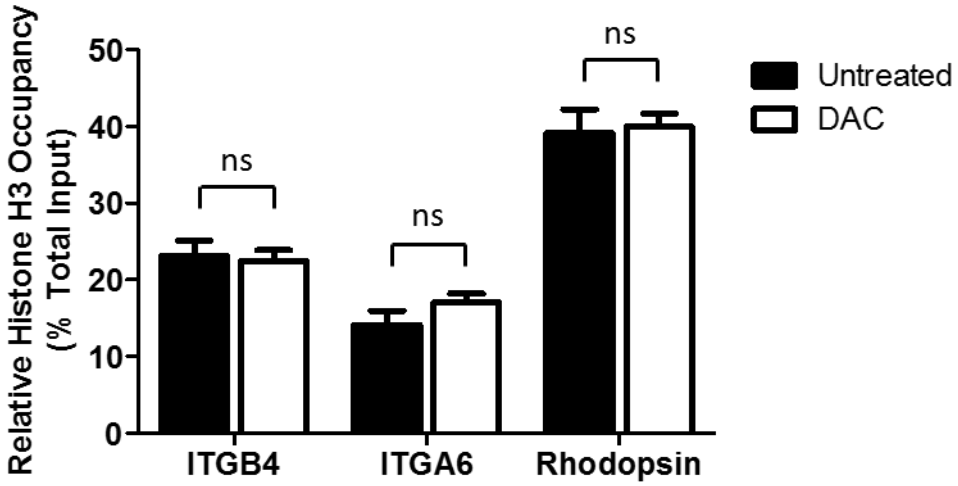


Figure 5.11 – Effect of DAC treatment on histone H3 and histone H3 acetylation ITGB4 and ITGA6 promoters in KG-1a cells. ChIP assays were performed in untreated and DAC treated KG-1a cells with an antibody against **A)** acetyl-H3 and **B)** histone H3. Immunoprecipitated DNA was analysed by qPCR with primers that amplify a region of ITGB4, ITGA6 and Rhodopsin promoters. The data are shown as the ratio of immunoprecipitated DNA to total input DNA. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using Students' *t* Test, **p*<0.05, ns *p*>0.05.

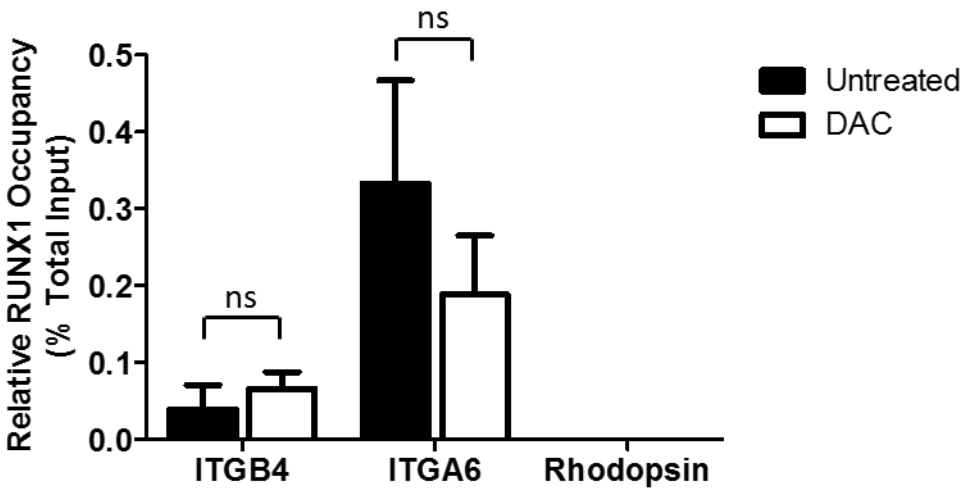


Figure 5.12 – RUNX1 binding at ITGB4 and ITGA6 promoters in untreated and DAC treated KG-1a cells. ChIP assays were performed in untreated and DAC treated KG-1a cells with an antibody against RUNX1. Immunoprecipitated DNA was analysed by qPCR with primers that amplify a region of ITGB4, ITGA6 and Rhodopsin promoters. The data are shown as the ratio of immunoprecipitated DNA to total input DNA. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using Students' *t* Test, ns $p>0.05$.

5.3 Discussion

It is evident now that epigenetic mechanisms play a key role in the regulation of genes and are commonly disrupted in cancer. Data presented here suggest that integrin genes ITGB4 and ITGA6 are regulated by DNA methylation, histone acetylation and histone occupancy, and changes to these epigenetic components contributes to the differential expression observed in the leukaemic cell lines (as summarised in Table 5.2).

DNA methylation is a well characterised epigenetic modification and at the promoter of genes is commonly associated with gene repression or gene silencing (Sharma *et al.* 2010). Evidence presented here suggests that the ITGB4 gene is regulated by DNA methylation. Expression of *ITGB4* in KG-1a and Kasumi-1 cells was inversely correlated with methylation of the large CpG island present at the promoter. The low expression of *ITGB4* in KG-1a cells was associated with a higher level of methylation at the promoter, while higher expression of *ITGB4* in Kasumi-1 cells was associated with an unmethylated promoter and methylated gene body. These data are consistent with a previous study which found that DNA methylation of the Itgb4 promoter in mouse mammary gland cells is inversely correlated with expression of *Itgb4* in these cells (Yang *et al.* 2009). In contrast, *ITGB4* expression in K562 cells was not correlated with DNA methylation of the ITGB4 promoter, as the promoter was unmethylated in these cells. These data therefore suggest that K562 cells may be missing a factor that is required for the expression of the endogenous ITGB4 gene.

While *ITGA6* is expressed at higher levels in both KG-1a and Kasumi-1 cells, and lower levels in K562 cells, the ITGA6 promoter was found to be unmethylated in all cell lines examined in this study. Therefore, from this data it is not clear whether ITGA6 is regulated by DNA methylation. However, the ITGA6 gene possesses some gene body DNA methylation in K562 cells. Recent studies have indicated that intragenic CpG methylation regulates the use of alternative promoters in gene bodies, therefore leading to the expression of variant transcripts (Bert *et al.* 2013, Maunakea *et al.* 2010). The production of variant transcripts provides another mechanism of gene regulation in a cell, and commonly arise from alternative splicing of pre-mRNA and/or through the use of alternative promoters (Black 2003). Variant transcripts can give rise to protein isomers which differ in chemical and biological activity (Black 2003). To determine if *ITGA6* has an alternative promoter, FANTOM5 data, which has information on the location and

usage of transcription start sites along the genome in human cells and mouse primary cells, cell lines and tissues, and is available from the FANTOM5 project (Lizio *et al.* 2015), was interrogated. These data showed that *ITGA6* has many alternative transcription start sites, however, interestingly, there is a region located approximately +7.1 kb downstream of *ITGA6*, which possesses alternative transcription start sites (Figure 5.13). Furthermore, interrogation of ChIP-seq data from studies described in *Chapter 3* (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Ptasinska *et al.* 2014, Tijssen *et al.* 2011, Trombly *et al.* 2015) showed RUNX1 binding at this region in CD34+ cells, SKNO-1 cells and Kasumi-1 cells, and RUNX1-ETO binding in Kasumi-1 cells (Figure 5.13). Interestingly, within this region there is also a consensus RUNX1 binding motif located +7,391 to +7396 bp downstream of *ITGA6*. Taken together, the ITGA6 gene body methylation observed in K562 cells may span to a region approximately +7.1 kb downstream of *ITGA6* and block transcription factors from binding to an alternative promoter located within this region, therefore, inhibiting the expression of a variant transcript. Since the primers used for RT-qPCR analysis in this study were designed in the last exon of the full-length ITGA6 mRNA and can detect transcript variants 1, 2 and 3 (Accession number: NM001079818, NM00210 and NM001316306, respectively), the primers may also be able to detect the variant transcript produced from the use of the alternative promoter and therefore expression of this variant transcript cannot be distinguished from the other transcripts detected in this analysis. Further studies are therefore required to determine if the leukaemic cell lines produce a different ITGA6 transcript and whether expression of this transcript is inhibited in K562 cells.

While DNA methylation plays a critical role in regulating gene expression, modification to the histone proteins associated with the DNA is also an important epigenetic regulatory mechanism. The accessibility of a gene to its regulators in the nucleus is important for optimal expression within a cell. Nucleosomes, which are comprised of histone proteins, are not only important for packaging of DNA within a cell, but also play a key role in regulating the accessibility of genes. Additionally, acetylation of these histone proteins at the promoters of genes is strongly associated with transcriptional activation (Virani *et al.* 2012). In the present study, both ITGB4 and ITGA6 integrin gene expression was influenced by both histone occupancy and histone acetylation at their promoters in KG-1a and/or Kasumi-1 cells, with higher gene expression associated with decreased histone occupancy and increased histone acetylation. Not surprisingly, these data suggest that for

optimal expression of these genes, the promoters must be accessible to regulatory proteins.

Epigenetic inhibitors, such as the demethylating agent DAC, are a useful tool to study the regulation of genes by epigenetic modifications. In this study, treatment of myeloid cell lines K562, KG-1a and Kasumi-1 with DAC increased *ITGB4* expression, while *ITGA6* expression only increased in the K562 cells. However, the increase of *ITGB4* and *ITGA6* expression in these cell lines, particularly K562 and KG-1a, was not due to demethylation of their promoter, suggesting that DAC had an indirect effect on these genes. This may have been due to the activation of genes encoding other transcription factors required for the expression of *ITGB4* and *ITGA6*, or alternatively, data presented here suggest that DAC can affect other epigenetic modifications. DAC was shown to increase histone acetylation at the promoters of *ITGB4* and *ITGA6*, which may have contributed to increased expression of these genes. This is supported by a previous study which found that the demethylating agent 5-azacytidine can alter genomic histone modification patterns (Komashko and Farnham 2010). Certain genes were shown to switch repressive histone marks from histone H3K27me3 to histone H3 lysine 6 trimethylation (H3K9me3) and interestingly, most genes which displayed altered expression did not possess DNA methylation at their promoters prior to treatment (Komashko and Farnham 2010). Taken together, these data suggest that DAC may have increased *ITGB4* and *ITGA6* expression in myeloid cells indirectly by altering histone acetylation as well as other histone modifications at the promoters of these genes.

Interestingly, while DAC did not cause demethylation of ITGB4 and ITGA6 promoters in most of the cell lines examined in this study, unexpectedly, DNA methylation increased in many of the clones analysed using bisulphite sequencing. The increase in DNA methylation after treatment with DAC has also been observed in a previous study (Chowdhury *et al.* 2015). It was found that while DAC causes decreased levels of methylated cytosines (5mC), it increases 5-hydroxymethylcytosine (5hmC) (Chowdhury *et al.* 2015). 5hmC is an intermediate of the demethylation process via the oxidation of 5mC by the ten-eleven-translocation (TET) DNA dioxygenases (Tahiliani *et al.* 2009). TET DNA dioxygenases play an important role in gene regulation for early embryonic development (Ficz *et al.* 2011), as well as differentiation of haematopoietic cells by priming genes for expression which regulate myeloid and lymphoid lineage commitment (Caron *et al.* 2015, Madzo *et al.* 2014, Tekpli *et al.* 2016, Tsagaratou *et al.* 2014). The

increase in 5hmC with DAC treatment is thought to result from TET enzymes targeting hemi-methylated DNA (Chowdhury *et al.* 2015). Furthermore, although 5hmC reacts with bisulphite and is converted to 5-methylenesulphonate while 5mC is resistant to bisulphite conversion, 5hmC cannot be distinguished from 5mC using bisulphite sequencing as they are both recognised as cytosines after treatment (Huang *et al.* 2010). Interestingly, DNA which possess a large fraction of 5-methylenesulphonate after bisulphite treatment is less efficiently amplified in PCR, as 5-methylenesulphonate sites are able to stall the Taq polymerase (Huang *et al.* 2010). Taken together, with the observation that *ITGB4* and *ITGA6* promoter regions were difficult to amplify by PCR and that there was also an increase in methylation at those regions in K562 and KG-1a cells, it might suggest that DAC treatment may have increased 5hmC during the de-methylation process. Further studies using different sequencing approaches such as pyrosequencing and high-throughput sequencing, which can distinguish 5hmC from 5mC (de la Rica *et al.* 2016), are therefore required to determine if 5hmC levels increase at ITGB4 and ITGA6 promoters in DAC treated leukaemic cell lines.

In addition to the DNA methylation inhibitor DAC, leukaemic cell lines were also treated with the histone acetyltransferase inhibitor TSA. While data presented here suggest that histone acetylation at the promoter is important for both *ITGB4* and *ITGA6* expression, only a combined treatment of DAC and TSA showed a significant increase in *ITGB4* expression, while TSA had no effect on *ITGA6* expression. This is in contrast to previous studies which have shown a decrease in both *ITGB4* and *ITGA6* expression upon treatment with the HDAC inhibitor valproic acid in PC-3 cells (Hudak et al. 2012, Wedel et al. 2011) and a decrease in *ITGA6* expression upon treatment of a hepatocellular carcinoma cell line with TSA (Lin *et al.* 2005). While it is expected that treatment of cells with HDAC inhibitors should increase the expression of genes regulated by histone acetylation, the decrease in *ITGB4* and *ITGA6* expression observed in these studies may be due to an indirect effect via the activation of repressors, which regulate *ITGB4* and *ITGA6*. Additionally, in these previous studies (Hudak *et al.* 2012, Lin *et al.* 2005, Wedel *et al.* 2011) cells were treated for much longer periods from 1-5 days, whereas in the present study cells were only treated for 6 hours. Longer treatment times may therefore result in indirect effects on expression of the integrin genes. Additionally, a previous study has observed that TSA treatment does not always result in increased histone acetylation at gene promoters and also does not always inhibit histone deacetylation (Mulholland *et al.* 2003).

In conclusion, data presented here provides evidence that both *ITGB4* and *ITGA6* are regulated by epigenetic mechanisms in myeloid cells. *ITGB4* is regulated by DNA methylation of a large CpG island located at its promoter, and both *ITGB4* and *ITGA6* expression is dependent on having an accessible promoter, due to decreased histone H3 and increased histone acetylation. In addition, the present study has further elucidated the effects of the de-methylating agent DAC, which is currently being used as a treatment for leukaemia (Blum *et al.* 2010, Cashen *et al.* 2010, Issa *et al.* 2004). DAC is thought to demethylate the whole genome, however evidence from previous studies and the current study suggest that its effects on the genome are more complex than previously thought and can lead to changes in other epigenetic modifications, including histone acetylation and methylation, and DNA hydroxymethylation (Chowdhury *et al.* 2015, Hagemann *et al.* 2011, Komashko and Farnham 2010).

	ITGB4			ITGA6		
	KG-1a	**Kasumi-1**	**K562**	**KG-1a**	**Kasumi-1**	**K562**
Normal expression	Low	Medium	Low	High	Medium	Low
Response to DAC	↑	↑	↑	No Change	No Change	↑
Response to TSA	↑	↑	↑	No Change	No Change	No Change
DNA methylation at the promoter	High	Low	Low	Low	Low	Low
Histone occupancy at the promoter	High	Low	-	Low	Low	-
Histone acetylation at the promoter	Low	-	-	High	-	-

Table 5.2 – Epigenetic modifications to *ITGB4* and *ITGA6* in myeloid cell lines. This table summarises the expression of *ITGB4* and *ITGA6*, as well as the DNA methylation, histone occupancy and histone acetylation status at the promoters of ITGB4 and ITGA6 in KG-1a, Kasumi-1 and/or K562 cells. The change in gene expression is also shown in response to treatment with DAC or TSA (arrow indicates an increase in gene expression).

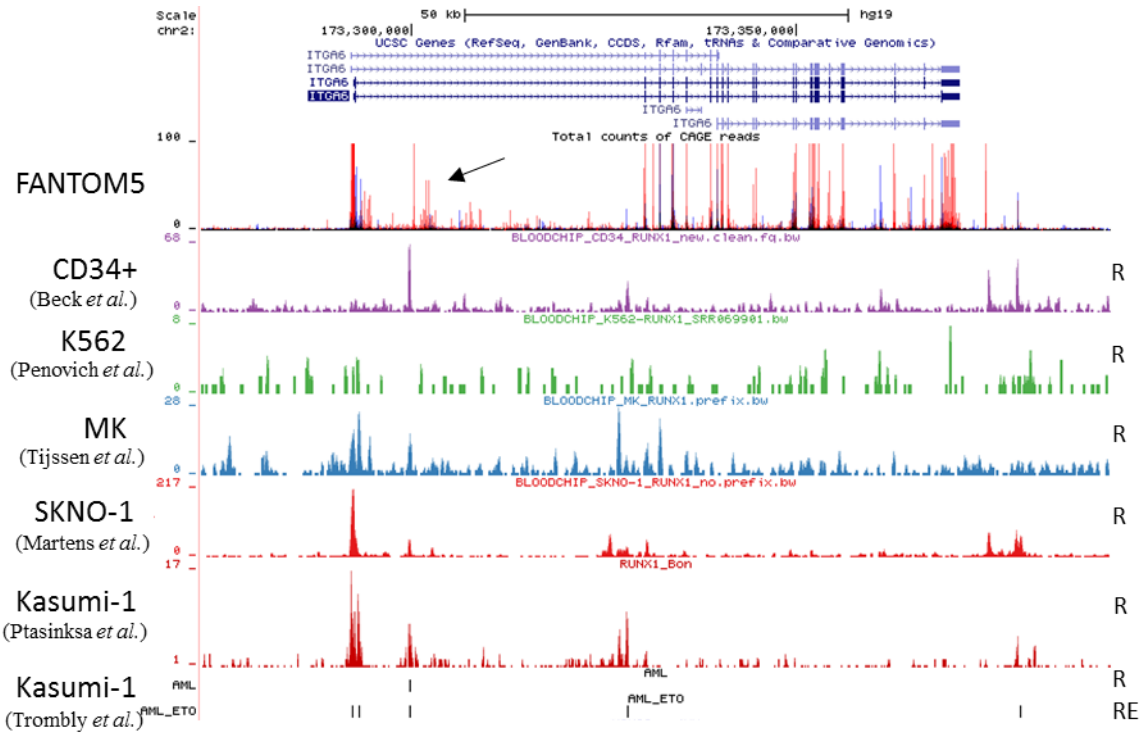


Figure 5.13 – Alternative transcription start sites for *ITGA6*. Screen shot of FANTOM5 data from the FANTOM5 project (Lizio *et al.* 2015) and data from ChIP-seq studies (Beck *et al.* 2013, Martens *et al.* 2012, Pencovich *et al.* 2011, Ptasinska *et al.* 2014, Tijssen *et al.* 2011, Trombly *et al.* 2015) visualised in UCSC Genome Browser (Kent *et al.* 2002). Peaks in the FANTOM5 data represent transcription start sites used in human cells and mouse primary cells, cell lines and tissues, and the arrow indicates the region that possesses alternative transcription start sites located approximately +7.1 kb downstream of *ITGA6*. Peaks and lines in the ChIP-seq data represent RUNX1 (R) and RUNX1-ETO (RE) binding in the different cell types. The ITGA6 gene is represented at the top of the figure, with exons shown as bars. The major transcript is highlighted in blue.

Chapter 6

Final Conclusions and Future Directions

Disruption to regulatory mechanisms controlling gene expression is a hallmark of leukaemia, with disruption to transcription factors being one of the most prevalent (Brettingham-Moore *et al.* 2015, Döhner and Döhner 2008). By identifying the gene expression profile under the control of these transcription factors, and understanding how the target genes are regulated, critical insight can be gained into the role of these transcription factors in haematopoiesis, as well as their role in leukaemia development. Additionally, this may identify potential biomarkers or therapeutics targets for the disease. In this study, the integrin genes ITGA6 and ITGB4 were identified as novel target genes of RUNX1.

RUNX1 is commonly described as a sequence-specific DNA binding transcription factor which binds to the promoters of its target genes and regulates their transcriptional activity (Meyers *et al.* 1993, Takahashi *et al.* 1995). However, it is evident now that RUNX1 regulation of gene expression is more complex than this, encompassing multiple regulatory layers involving interaction with other co-factors or transcription factors, distal regulatory elements and epigenetic factors (Bowers *et al.* 2010, Elagib *et al.* 2003, Huang *et al.* 2009, Kitabayashi *et al.* 1998, Levantini *et al.* 2011, Reed-Inderbitzin *et al.* 2006, Waltzer *et al.* 2003, Wotton *et al.* 1994, Zhang *et al.* 1996). Data presented in this thesis identified two distinct mechanisms by which RUNX1 regulates its target genes, as demonstrated by the ITGA6 and ITGB4 integrin genes. In addition, epigenetic modifications also play an important role in regulating their expression.

RUNX1 was demonstrated to regulate the ITGA6 promoter via the well described traditional model of RUNX1 function. RUNX1 was found to bind to a RUNX1 consensus motif within the ITGA6 promoter, as mutation to this site resulted in a significant reduction in RUNX1 activation of the promoter in reporter assays. In contrast, RUNX1 regulation of the ITGB4 promoter was found to be more complex. The data presented here suggest that RUNX1 may regulate the ITGB4 promoter indirectly through interactions with other haematopoietic transcription factors which may potentially bind

to the promoter. Additionally, RUNX1 may require an upstream enhancer to regulate the ITGB4 promoter, and may also interact with this region indirectly by binding to other haematopoietic transcription factors.

A further regulatory layer in ITGA6 and ITGB4 gene expression was epigenetic modifications. Data presented in this thesis suggest that promoter accessibility is important for the expression of *ITGA6* and *ITGB4*, as expression of these genes was higher in leukaemic cells with lower occupancy of histone H3 and higher levels of histone acetylation at the promoter regions. The data also suggest that the expression of *ITGB4* is influenced by DNA methylation of a CpG island present at the promoter. In two of the three myeloid cells analysed in this study, DNA methylation levels at the ITGB4 promoter was inversely correlated with expression of this gene.

While it is clear that the regulation of ITGA6 and ITGB4 is multi-layered, including transcriptional and epigenetic factors, there is interplay between these different layers. There is evidence in the literature that RUNX1 can also influence gene transcription through interaction with epigenetic modifiers that drive epigenetic changes at target genes. The outcome of gene activity is therefore dependent on the balance of activating and repressive factors associated with RUNX1, as reviewed in (Brettingham-Moore *et al.* 2015). For example, to repress gene expression RUNX1 can interact with the repressive factors mSin3A, SUV39H1 and HDACs to create condensed chromatin via histone deacetylation and DNA demethylation (Lutterbach *et al.* 2000, Reed-Inderbitzin *et al.* 2006). However, RUNX1 can also activate gene expression by interacting with histone acetyltransferases p300 and CBP (Kitabayashi *et al.* 1998, Oakford *et al.* 2010). Furthermore, a recent study has demonstrated RUNX1 binding to be associated with increased histone acetylation in haematopoietic development (Lichtinger *et al.* 2012). The presence of histone acetylation at the ITGA6 and ITGB4 promoters in leukaemic cell lines in this study may therefore be facilitated by RUNX1 and/or other transcription factors complexing with RUNX1.

Additionally, the RUNX1 protein itself can be modified by epigenetic factors which can alter its activity on its target genes. Acetylation of RUNX1 by p300 can increase its DNA binding and transcriptional activity (Oakford *et al.* 2010, Yamaguchi *et al.* 2004). Furthermore, methylation of RUNX1 by the histone methyltransferase PRMT1 results in increased RUNX1 transcriptional activity due to the disruption to the interaction of

RUNX1 and the co-repressor complex involving mSin3A (Zhao *et al.* 2008). In contrast, interaction of RUNX1 with the histone methyltransferase SUV39H1 results in inhibition of RUNX1 activation of target genes due to decreased binding of RUNX1 at gene promoters (Chakraborty *et al.* 2003). Additionally, methylation by the histone methyltransferase PRMT4 also leads to gene repression due to the formation of a methyl-RUNX1-dependent co-repressor complex (Vu *et al.* 2013). Further studies are therefore required to determine if there is interplay between RUNX1 and epigenetic factors at the ITGA6 and ITGB4 promoters.

In the present study, leukaemic cell lines K562, KG-1a and Kasumi-1 cells were used as a model system to elucidate the transcriptional and epigenetic regulation of integrin genes ITGA6 and ITGB4. These cell lines have been useful in dissecting the mechanisms of regulation of *ITGA6* and *ITGB4* as they display differential expression of these genes. While it is clear that epigenetic mechanisms have an effect on *ITGA6* and *ITGB4* expression in the leukaemic cell lines KG-1a and Kasumi-1, low expression of *ITGA6* and *ITGB4* was not correlated with DNA methylation in K562 cells. It was hypothesised that the low expression of *ITGA6* and *ITGB4* in K562 cells may be due to low levels of RUNX1 present in these cells, however, overexpression of RUNX1 in K562 cells only resulted in an increase in *ITGA6* expression (data not shown). These data therefore suggest that the K562 cells may not express a co-factor or transcription factor which is required for the expression of *ITGB4*.

Data presented here suggest that RUNX1 may regulate the ITGB4 promoter in a large transcription factor complex with other haematopoietic transcription factors such as E2A, SCL, FLI1, ERG, GATA1/GATA2, LYL1 and LMO2. RUNX1 regulation of *ITGB4* may therefore be dependent on the presence of these transcription factors. To determine if one of these haematopoietic transcription factors may be missing in K562 cells, expression data from published microarray studies were interrogated (de Jonge *et al.* 2011, Johnson *et al.* 2010, Ptasinska *et al.* 2012, Shia *et al.* 2012). Interestingly, the FLI1 transcription factor is expressed at very low levels in K562 cells (Figure 6.1), while other transcription factors such as E2A, SCL, ERG, GATA1 and LMO2 are present (GATA2 and LYL1 expression was not analysed in this data). In support of these findings, other studies have also detected either no or very low levels of FLI1 in K562 cells (Athanasiou *et al.* 1996, Watson *et al.* 1992). Interestingly, FLI1 plays an important role in haematopoietic cell differentiation through both positive and negative regulation, and has been shown to act

synergistically with GATA1 to regulate megakaryocyte-specific genes (Athanasiou *et al.* 2000, Eisbacher *et al.* 2003). Furthermore, treatment of K562 cells with PMA results in increased expression of FLI1 and overexpression of FLI1 in K562 cells causes changes similar to what is observed with PMA treatment, such as altered cell morphology and increased adherence (Athanasiou *et al.* 1996). Therefore, increased expression of *ITGB4* in PMA treated K562 cells, described in *Chapter 3*, may not only be due to activation of RUNX1 via phosphorylation as hypothesised, but may also be due to increased expression of FLI1. Interestingly, FLI1 has been shown to bind to the ITGB4 promoter in the leukaemic cell line U937, suggesting that it has the potential to bind to *ITGB4* in other cell types and further supporting its involvement in regulating *ITGB4* expression (Martens *et al.* 2012). While RUNX1 was shown to activate the ITGB4 promoter in reporter assays in K562 cells in the absence of FLI1, this overexpression of RUNX1 to abnormal levels may artificially force DNA-protein interactions, allowing RUNX1 to activate the promoter without FLI1. Alternatively, FLI1 may be important for the promoter-enhancer interaction highlighted in this study which may be required for RUNX1 regulation of the endogenous ITGB4 gene. If this were the case the absence of FLI1 in reporter assays would not have an effect on RUNX1 activation of the promoter alone.

Taking the results presented here, a model for the regulation of *ITGB4* in different cell lines has been proposed (Figure 6.2). This model proposes that *ITGB4* is expressed in Kasumi-1 cells due to the presence of all transcription factors required for the transcription of the gene and an unmethylated promoter. In KG-1a cells, although the transcription factors required for expression may be present, *ITGB4* is expressed at low levels, and this may due to high levels of DNA methylation and reduced accessibility at the promoter, which is preventing transcription factors from binding. In K562 cells, *ITGB4* is expressed at low levels although the promoter is unmethylated, this model proposes that this is because these cells are missing the FLI1 transcription factor. These findings suggest that for efficient expression of *ITGB4*, the promoter must be unmethylated and at least RUNX1 and FLI1 must be expressed. Additionally, although Kasumi-1 cells possess the RUNX1-ETO fusion protein, *ITGB4* is expressed at higher levels in these cells. However, as discussed in *Section 3.3* (Chapter 3), the variant transcript RUNX1-ETO9a, which is expressed in Kasumi-1 cells, can act as a strong activator due to the absence of the C-terminal inhibitory domains (Yan *et al.* 2004, Yan *et al.* 2006). In this model, RUNX1-ETO9a may also interact with these haematopoietic

transcription factors in Kasumi-1 cells, causing increased expression of *ITGB4* in these cells. Further studies would therefore be required to determine if FLI1 is a critical factor in the regulation of *ITGB4* and to determine if RUNX1-ETO9a regulates *ITGB4* similarly to RUNX1.

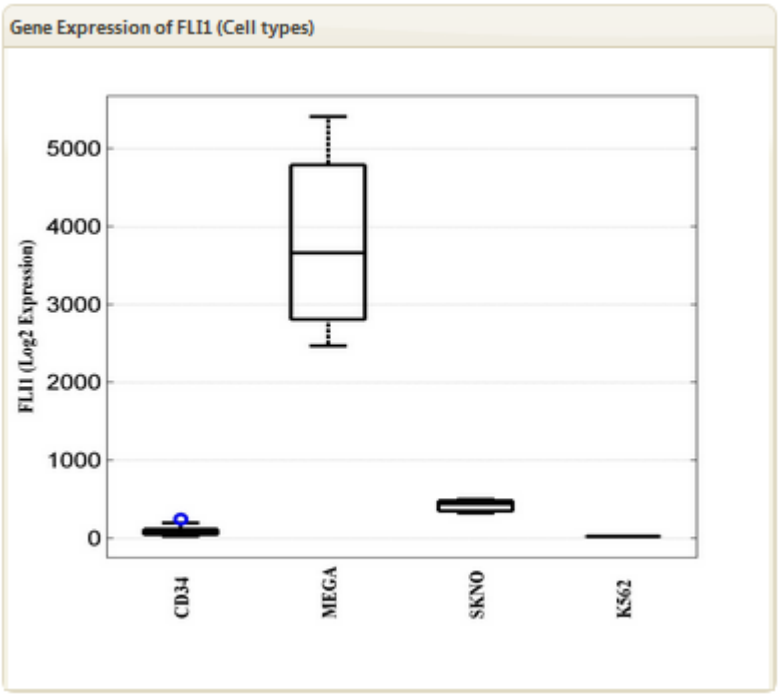


Figure 6.1 – FLI-1 expression in haematopoietic cells. Screen shot of FLI1 expression in CD34+ cells (GSE30029), megakaryocytes (E-TABM-633), SKNO-1 cells (GSE34594) and K562 cells (GSE28135) from genome-wide microarray expression data (de Jonge *et al.* 2011, Johnson *et al.* 2010, Ptasinska *et al.* 2012, Shia *et al.* 2012) viewed in the BloodChIP database (Chacon *et al.* 2014). Non-normalised expression data from illumina expression arrays were log2 transformed and quantile normalised. Range of FLI1 expression for 3-31 replicates is shown, as well as the mean expression (horizontal line) and standard deviation (whiskers).

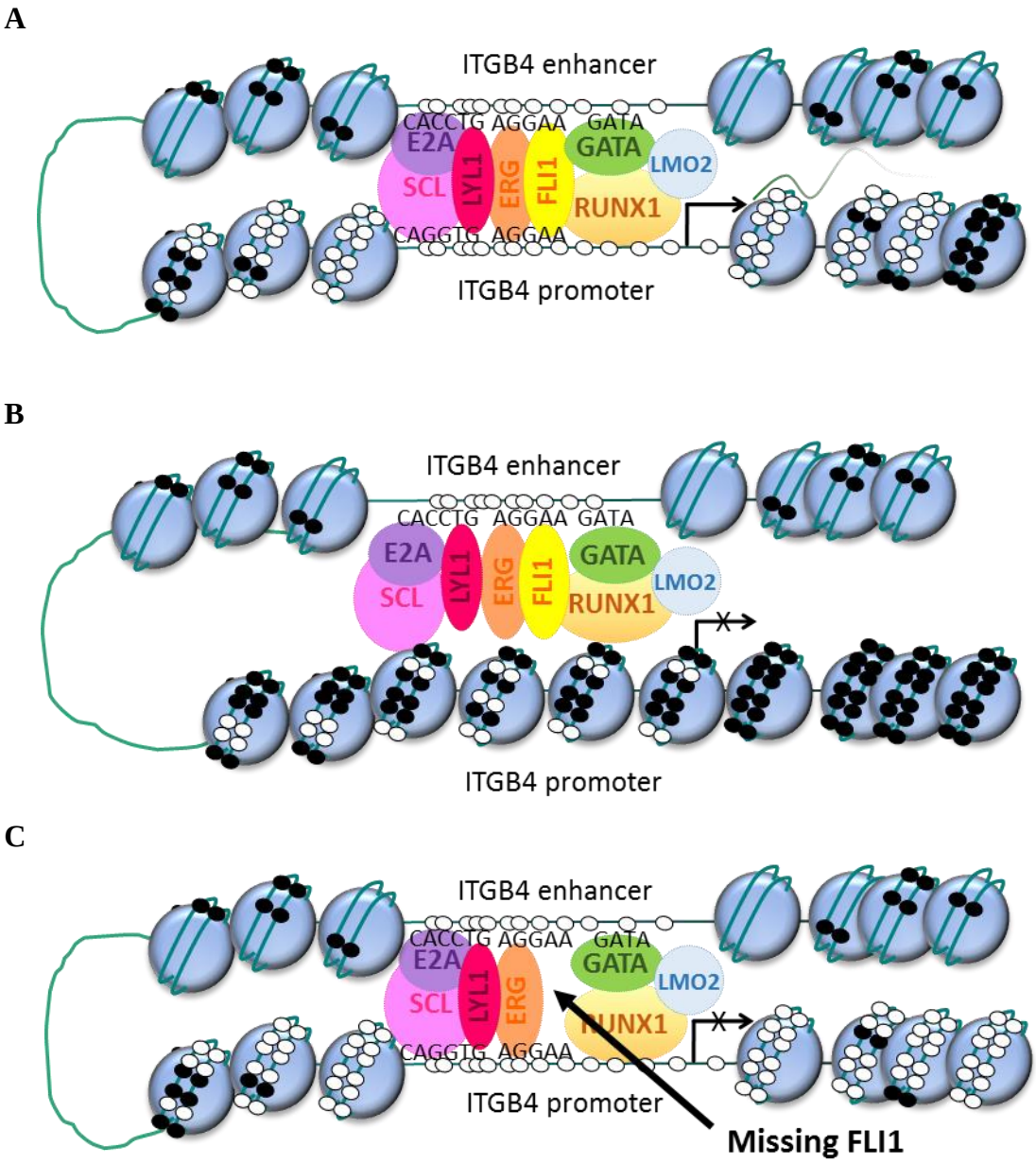


Figure 6.2 – A model for the regulation of *ITGB4* in different leukaemic cell lines. Depiction of *ITGB4* regulation in **A)** Kasumi-1, **B)** KG-1a and **C)** K562 cells. DNA is shown as aqua lines wrapped around nucleosomes (blue balls) and CpG sites are shown as small circles either methylated (black) or unmethylated (white). Transcription start sites are shown as black right-angled arrows. The ITGB4 promoter is unmethylated in Kasumi-1 cells, therefore allowing the binding of the transcription factor complex and interaction with the upstream enhancer, resulting in the expression of *ITGB4*. The ITGB4 promoter is methylated in KG-1a cells which causes tight packing of DNA, therefore necessary transcription factors are unable to easily bind to the ITGB4 promoter and results in little/no expression. The ITGB4 promoter is unmethylated in K562 cells, therefore allowing transcription factors to bind, however, FLI1 is not expressed in these cells, which may lead to a less efficient assembly of the transcription factor complex at the ITGB4 promoter. Therefore, the absence of FLI1 may be the cause of low expression of *ITGB4* in K562 cells.

Although the traditional model of RUNX1 regulation of genes via a RUNX1 consensus motif is well described in the literature and was identified in this study to be the mechanism by which RUNX1 regulates *ITGA6*, recent evidence suggests that this mechanism only represents a small proportion of RUNX1 target genes. Analysis conducted by our research group using ChIP-seq data from the study by Trombly *et al*. (2015) determined the proportion of RUNX1 binding in either promoter or intragenic/intergenic regions across the genome and which occurred in the presence of a RUNX consensus binding motif (Woodworth and Holloway, unpublished). Interestingly, only 21% of RUNX1 binding was located in promoter regions of genes while the other 79% was located in intragenic or intergenic regions. These findings are supported by other ChIP-seq studies which have also observed a higher percentage of RUNX1 binding in intragenic/intergenic regions compared to promoter regions (Beck *et al.* 2013, Wilson *et al.* 2010). Unexpectedly, of the sites found to be bound by RUNX1 in promoter regions, only 57% contained a RUNX motif. Together, these data suggest that the mechanism by which RUNX1 regulates *ITGB4* may occur more frequently than the traditionally described mechanism in which RUNX1 regulates expression via a binding motif in a promoter, as found here for *ITGA6*. It also suggests that the way in which RUNX1 is disrupted in leukaemia may therefore affect its target genes differently. For example, if there is a mutation which disrupts the DNA binding domain of RUNX1, this may affect the expression of genes that are regulated by RUNX1 through its binding to RUNX consensus sequences, such as *ITGA6*, differently to genes such as *ITGB4*, where RUNX1 is recruited independently of a canonical sequence motif. This therefore warrants further investigation.

The different mechanisms by which RUNX1 can regulate genes identified in this study, advances our knowledge of transcriptional regulation. While these mechanisms are true for RUNX1, they may also be relevant for other transcription factors. Interestingly, recent studies of other transcription factors such as Myc and NF-κB, have similarly found a significant proportion of binding occurs independently of DNA binding motifs (Guo *et al.* 2014, Kolovos *et al.* 2016). In addition, these findings may have implications for other types of cancer. The mechanisms described here for RUNX1 may also apply to the RUNX family members, RUNX2 and RUNX3, which are often disrupted in prostate, breast, gastric and brain cancer.

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